Interventions in the metabolic syndrome: bile acid sequestration and exercise

Maxi Meißner

Paranimfen **Gemma Brufau**
Milica Stankovic

The research described in this thesis was carried out at the Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen and was funded by an unrestricted grant from Kruidvat (Hutchinson Whampoa Ltd) and University of Groningen

The author gratefully acknowledges the financial support for printing of this thesis by:
Rijksuniversiteit Groningen
Faculteit der Medische Wetenschappen
Groningen University Institute for Drug Exploration
Conny Kirsch
Fresenius Kabi Nederland B.V.
Buchem bv
Dr. Falk Pharma GmbH

Colofon
Layout: Maxi Meissner
Cover design: “Gallsäure-und Cholesterol im Tanz” by Yvonne Mielecke (yvonne.mielecke@web.de) and Maxi Meissner (idea)
Printer: Ipskamp Drukkers B.V., Enschede

ISBN: 978-90-367-4602-1

RIJKSUNIVERSITEIT GRONINGEN

Interventions in the metabolic syndrome: bile acid sequestration and exercise

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 24 november 2010
om 14:45 uur

door

Maxi Meissner

geboren op 21 april 1980
te Berlin, Duitsland

Promotores:	prof. dr. F. Kuipers prof. dr. A.K. Groen
Beoordelingscommissie:	prof. dr. G. van Dijk prof. dr. P.J.J. Sauer prof. dr. B. Staels

Paved roads may never command over
the rhythm of your stride and
the beating of your heart.

CONTENTS

CHAPTER 1

Thesis Introduction

INTRODUCTION

The prevalence and severity of obesity have been increasing in children, adolescents and adults of developed countries [1-3]. Obesity during childhood and adolescence predisposes to adult obesity and is associated with various cardiovascular co-morbidities in adult life [4]. Many of these cardiovascular co-morbidities are related to anthropometric and metabolic imbalances that jointly characterize the metabolic syndrome (MetS) [5]. Furthermore, the increasing prevalence of MetS in childhood and adolescence [6, 7] is alarming as it promotes premature development of cardiovascular disease in the pediatric population [8].

THE METABOLIC SYNDROME

Definition

The metabolic syndrome (MetS) represents a daunting global health and economic challenge as its prevalence has reached alarming dimensions [9-11] paralleling those of the obesity epidemic. In its early stages, MetS is characterized by a constellation of metabolic derangements, including obesity and insulin resistance, which over years may progress to more overt diseases such as type 2 diabetes and cardiovascular disease [12-16]. Although the concept of such a metabolic disease state has existed for over 80 years [17], it was not until 1998 that the array of metabolic derangements comprising the MetS was defined by the World Health Organization [17]. This initial definition of metabolic risk factors was further modified by the National Cholesterol Education Program's Adult Treatment Panel III to be easily applied in the clinical and research setting [18]. Herein, the MetS traits include abdominal obesity, hyperglycemia, insulin resistance, atherogenic dyslipidemia, hypertension, inflammation and impaired endothelial function [19, 20]. Affected individuals typically are at increased risk to develop type 2 diabetes and cardiovascular diseases and premature mortality [21, 22]. In fact, the most common complication of MetS is type 2 diabetes. Type 2 diabetes manifests by elevated plasma glucose levels underlying either the inability of tissues to clear glucose

from the blood or an increased hepatic glucose production or both. Cardiovascular disease is the major cause of morbidity and mortality in patients with type 2 diabetes and cardiovascular risk is two to fourfold increased in type 2 diabetics over non-diabetic subjects [23, 24]. Altogether, MetS represents a complicated pathological state comprised of risk factors that are interrelated and can act to induce serious cardiovascular complications over years (Figure 1).

The work in this thesis pertains to two major risk factors of the Mets, hypercholesterolemia and hyperglycemia. A short overview on these two risk factors is given subsequently. (For further reading over the other risk factors, refer to BOX 1.)

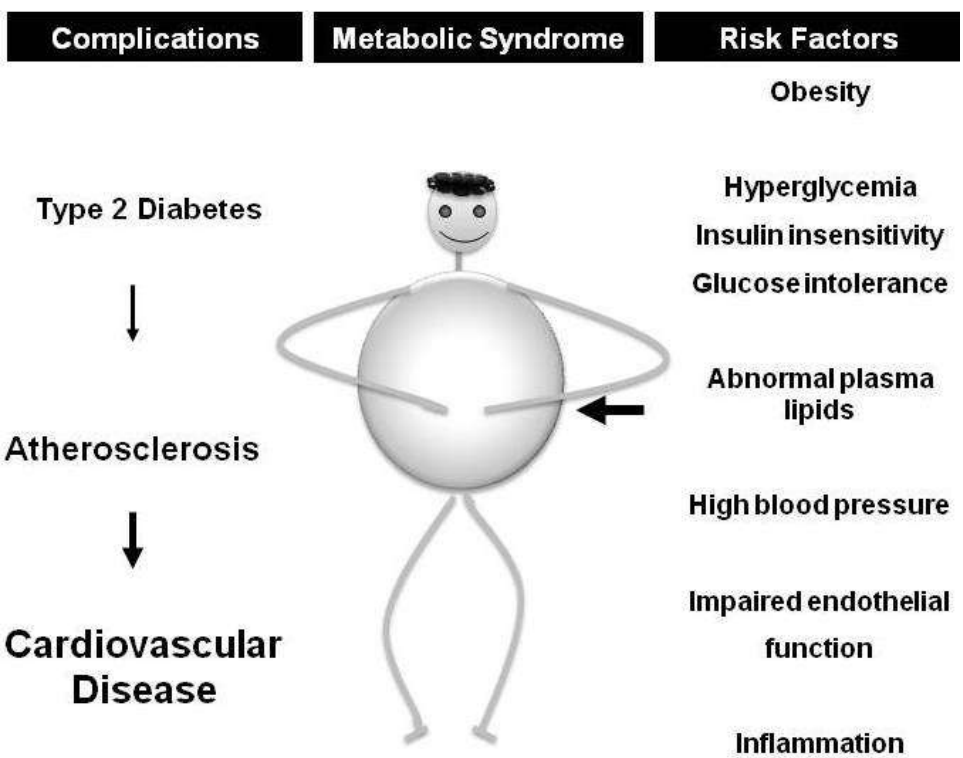


Figure 1. Risk factors and complications of the Metabolic Syndrome. The MetS risk factors are comprised of a variety of metabolic derangements which are interrelated and can act to induce cardiovascular disease.

Hypercholesterolemia as risk factor for the Metabolic Syndrome

Raised total cholesterol levels predispose to atherosclerosis, a disease characterized by inflammation of the vascular wall. Elevated blood cholesterol levels manifest as abnormalities in blood concentrations of lipoproteins. Lipoproteins are the carriers of cholesterol in the blood. High levels of low density lipoprotein (LDL) and small dense LDL are regarded pro-atherogenic, and in MetS LDLs are more easily oxidized [25]. Oxidized LDL stimulates an array of atherosclerotic processes, herein oxidized LDL is rapidly taken up by macrophages which promotes foam cell formation, oxidized LDL produces chemotractive effects on monocytes and stimulates cytokine production which promote an inflammatory state. LDL particles vary in size and density, the smaller and denser (i.e. containing fewer triglycerides but a higher proportion of cholesterol esters) the LDL particle is, the more atherogenic. For example, small dense LDL has less affinity for the LDLR and thereby increases formation of foam cells [26]. Moreover, small dense LDL has a higher susceptibility for oxidation [25] and it is believed to more easily damage the endothelium of the vessel wall [27]. Due to its adverse health effects, LDL has been termed the “bad cholesterol”. Based on numerous epidemiological studies, high levels of another lipoprotein, the high density lipoprotein (HDL) is believed to have anti-atherogenic properties [28] and thus, contrary to LDL, HDL has been termed the “good cholesterol”. One of those anti-atherogenic properties relevant to hypercholesterolemia is the role of HDL in the reverse cholesterol transport (RCT) pathway. RCT represents a major physiological pathway to clear excess cholesterol from the body. This pathway is comprised of a series of HDL-mediated steps that brings about removal of cholesterol from peripheral tissues, including cholesterol-laden macrophages in vessel walls, its subsequent transport to the liver, secretion into the bile as cholesterol or bile acid and further excretion into the feces [29, 30]. Thus, low HDL levels are generally considered to impair this hepatobiliary clearance route of cholesterol, although recent work indicates that this concept needs reconsideration.

Importantly, within the MetS risk stratification, hypercholesterolemia comprises part of atherogenic dyslipidemia, which is characterized by elevated plasma triglycerides levels. Hypertriglyceridemia leads to an increased catabolism of HDL. In short, hypertriglyceridemia promotes an increased transfer of triglycerides to HDL *via* cholesteryl ester transfer protein (CETP). This leads to the formation of triglyceride-rich HDLs which can be easily catabolized by hepatic lipase [26]. HDL particles have been shown to be more triglyceride-rich in insulin resistant and type 2 diabetic populations, where HDL levels are low [26].

Hyperglycemia as risk factor for the Metabolic Syndrome

Depending on the severity, hyperglycemia can be divided into either impaired fasting glycemia (8-hour fasting glucose levels ≥ 7.0 mmol/L) or type 2 diabetes (8-hour fasting glucose levels ≥ 11.1 mmol/L) [31]. The severity of hyperglycemia can change over time, reflecting the severity of underlying metabolic derangements. However, both conditions qualify as hyperglycemia and display undesirably high glucose levels, which chronically exert physiological stress, organ damage and dysfunction, especially to the eyes, kidneys, nerves, heart and blood vessels [31].

Underlying causes of hyperglycemia include obesity and abnormalities in adipose tissue [32], peripheral and hepatic insulin resistance, impaired beta-cell function which can all give rise to an abnormal hepatic glucose production and impaired peripheral and hepatic glucose uptake. Thus, the net effect is that either too much glucose is release into the blood by hepatic production, or the glucose cannot be cleared from the blood due to impaired uptake mechanisms of tissues, or both. For additional information on the relationships between obesity, insulin resistance and MetS, refer to BOX 1.

As the prevalence of MetS is dramatically increasing, so is the ever-increasing economic burden of the MetS and its associated complications like type 2 diabetes, atherosclerosis and cardiovascular disease. Cost-effective and efficient treatment strategies are in high demand. This thesis describes how alterations of bile acid metabolism by pharmaceutical or lifestyle intervention affect

hypercholesterolemia and hyperglycemia. We specifically address the effects of bile acid sequestration (BAS) on hyperglycemia and hypercholesterolemia and further investigate whether physical activity modulates bile acid metabolism and thereby cholesterol homeostasis. For these studies, well-defined (genetically engineered) mouse models were used.

BILE ACIDS AND THE METABOLIC SYNDROME

Bile acids activate a number of signaling pathways involved in the regulation of lipid and glucose, but also energy metabolism [33]. Their involvement in this wide array of metabolic pathways makes them attractive candidates for pharmaceutical intervention in the Mets where lipid, glucose and energy metabolism are imbalanced.

Bile acids: Function, metabolism and implication for Mets

Bile acids were originally identified as detergents to facilitate the digestion and absorption of fats and fat-soluble molecules in the intestine and to keep cholesterol from precipitating into the bile.

Bile acids are synthesized by enzymatic conversion from cholesterol in the liver. Additionally, with a species dependent ~10-100 times higher concentration in bile compared to cholesterol, bile acids also comprise the most sterol and sterol-derived compounds in bile. Thus, bile acids represent important end-products in cholesterol metabolism. The biosynthesis of bile acids is mediated by 2 pathways: the classic (or neutral) pathway and the alternative (or acidic) pathway, each involving a series of enzymatic steps [34]. The rate-controlling enzyme for the classic pathway is cholesterol 7 α-hydroxylase (CYP7A1) and that for the alternative pathway is sterol 27 hydroxylase (CYP27A1) [34]. Bile acids can regulate their own synthesis by controlling the expression of genes involved in their synthesis, thereby creating a feedback loop. Herein, the nuclear receptor farnesoid x receptor (FXR) has a central role; when activated by bile acids, FXR acts to

inhibit the bile acid synthetic pathway on various levels [33]. In short, activated hepatic FXR triggers small heterodimer partner (SHP) to inhibit the expression of CYP7A1 and thereby inhibit *de novo* bile acid synthesis. Moreover, activation of FXR by bile acids in the distal ileum induces the expression of fibroblast growth factor 15 (FGF15) which is subsequently released into the portal blood stream and acts to reduce the activity of hepatic CYP7A1 *via* binding to fibroblast growth factor 4/β-klotho receptor complex (FGFR4) in the liver and subsequent activation of signal transduction pathways including the JNK/ERK pathway. Contrarily, under situations of low bile acid availability, FXR is not active and these two pathways that prevent *de novo* bile acid synthesis are not activated thus allowing the biosynthesis of bile acids to increase.

Commonly referred to as the enterohepatic circulation, bile acids circulate between the liver and intestine by bile and portal blood (Figure 2). Ninety-six % of biliary bile acids is reabsorbed by active transport into the enterocytes of the terminal ileum, while a small fraction, ~4%, escapes absorption and is excreted into feces [34]. The body tries to keep the bile acid pool size constant as both the accumulation of bile acids in the body and a decreased bile acid pool can have serious pathophysiological consequences. Thus, any fraction of bile acids that escapes reabsorption is newly synthesized from cholesterol in the liver. Fecal bile acid secretion, therefore, equals *de novo* bile acid synthesis and thereby has a major contribution to cholesterol turnover. Intriguingly, perturbations that provoke an increase in fecal bile acid excretion induce an enhanced cholesterol turnover and thereby represent attractive treatment strategies for hypercholesterolemic patients.

Bile acid sequestration and cholesterol homeostasis

Bile acid sequestrants (BAS) are agents that induce an enhanced fecal bile acid excretion [35]. In fact, BAS have long been known for their cholesterol-

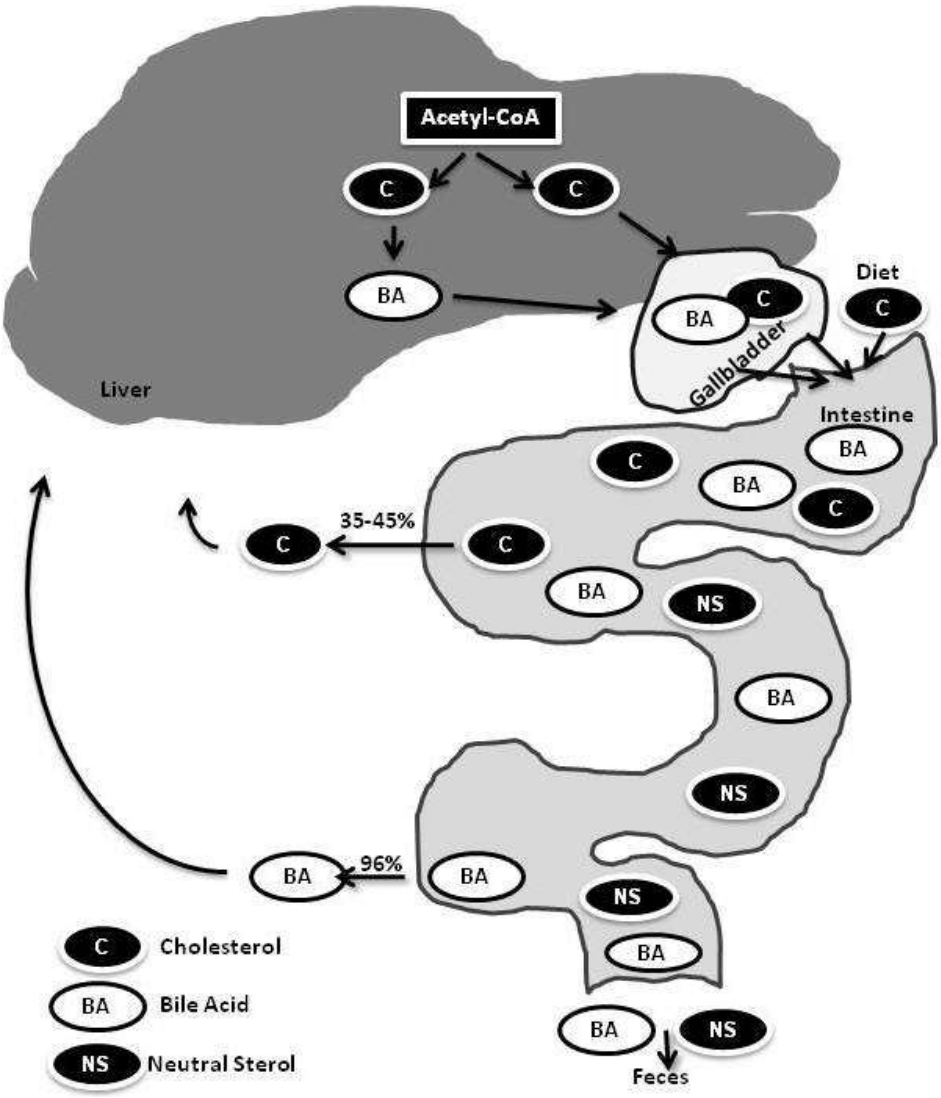


Figure 2. Enterohepatic cholesterol and bile acid metabolism. Cholesterol is either secreted from the liver into the gallbladder as cholesterol or as a bile acid after undergoing enzymatic conversion. Cholesterol and bile acids are transported across the canalicular membrane into the bile. The gallbladder secretes cholesterol and bile acids into the intestine, where bile acids mix with cholesterol to form micelles for cholesterol absorption. In the proximal intestine ~20-70% of cholesterol is absorbed into the intestinal lumen in humans (~35-45% in mice), subsequently expelled into the portal blood and returned to the liver. Cholesterol can also be secreted from the Most bile acids (~96%) are absorbed in the terminal ileum and recycled back to the liver (enterohepatic circulation), while the remaining 4% are excreted with the feces. To maintain the bile acid pool size in equilibrium, the amount of bile acids lost via the feces is compensated for by *de novo* bile acid synthesis from cholesterol in the liver.

lowering actions. The older generation BAS *e.g.,* cholestyramine and colestipol were the first choice treatment to reduce LDL cholesterol levels in patients with hyperlipidemia in the pre-statin era. Later, human studies have demonstrated that the BAS-induced decrease in total cholesterol and LDL cholesterol, were not only accompanied by considerable relative risk reductions in fatal and nonfatal myocardial infarction [36] and coronary heart disease death [37] but also by increased regression and decreased progression in coronary artery lesion [38-42]. Moreover, BAS have been shown to increase HDL levels slightly, by 4-8% [43].

BAS are positively charged non-absorbable resins; in the intestinal lumen BAS bind to negatively charged bile acids and bound to bile acids they are subsequently excreted into feces. By forming these non-absorbable complexes with bile acids, thereby disrupting the enterohepatic circulation of bile acids and provoking their malabsorption, BAS stimulate the liver to convert endogenous cholesterol into bile acids to maintain the bile acid pool size [44]. Thus, the BAS-induced bile acid malabsorption subsequently leads to an increased cholesterol conversion to bile acids. Herein, a compensatory increase in hepatic LDL receptor activity has been proposed to clear LDL cholesterol from the circulation, thereby reducing LDL cholesterol levels [45].

Although the cholesterol lowering actions of BAS are well established, it is not, yet, clear what the effect of BAS are on atherosclerotic lesion size development. Moreover, the exact effects of BAS on bile acid metabolism upon MetS are unknown, while also a thorough characterization of bile acid metabolism under MetS has not been established.

Bile acid sequestration and glucose homeostasis

In fact, recent evidence suggests that bile acid metabolism in itself is altered in type 2 diabetes [46], a complication of MetS. For example, an increased bile acid pool size has been reported for *db/db* mice [47] and liver-specific insulin receptor knockout mice [48], two widely utilized type 2 diabetic mouse models. Intriguingly, leptin administration to another type 2 diabetic mouse model, the *ob/ob* mouse has

shown to not only improve insulin sensitivity but also reduce the bile acid pool size [49]. Moreover, administration of insulin to cultured rat hepatocytes reduced expression of CYP7A1 and CYP27A1 [50]. These observations indicate a close link between hepatic insulin sensitivity and bile acid metabolism. Additionally, disrupting the enterohepatic circulation of bile acids by BAS was shown to benefit various parameters important for glycemic control, e.g., to reduce plasma glucose, glycosylated hemoglobin and the urinary glucose excretion but also to improve glycemic control and reduce the body mass index in patients displaying type 2 diabetes and dyslipidemia [35, 44, 46, 51-53]. Indeed, Colesevelam HCl, a second-generation BAS, has been indicated by the FDA to improve glycemic control in patients with type 2 diabetes [54-56]. Furthermore, administering BAS to a diabetic mouse model improved insulin resistance and fatty liver [57]. These data opened new perspectives for the treatment of MetS and cardiovascular disease risk factors.

The mechanisms behind the BAS-induced reduction in plasma glucose levels are so far unknown but are thought to be mediated through direct interference of BAS with bile acid signaling pathways. Bile acid signaling pathways involve nuclear receptors, like FXR, which also has an established role in glucose metabolism [47, 58]. Granted that bile acids by themselves have been found to mediate the regulation of hepatic gluconeogenesis [59], it appears possible that BAS might modulate glucose homeostasis by FXR-dependent signaling pathways, thereby regulating the expression of enzymes implicated in hepatic gluconeogenesis and glucose utilization. FXR-independent pathways might entail a BAS-induced secretion of insulino-tropic incretins such as glucagon-like-peptide-1 and gastric inhibitory polypeptide. By attaching to bile acids in the intestine, BAS are believed to interfere with proper chylomicron formation, thus preventing adequate intestinal fatty acid absorption thereby provoking an increased fatty acid-pass to more distal parts of the intestine where fatty acids can stimulate the release of GLP-1 [60] . However, the mechanisms concerning the glucose-lowering actions of BAS remain speculative to date.

It is noteworthy that next to their cholesterol-lowering and glucose-lowering actions BAS have additional therapeutic effects specific for other risk factors of MetS (*Figure 3)*. Those pleiotrophic effects entail improvements in energy metabolism [35, 44, 46, 51-53, 61] and inflammation [35, 51, 62, 63] as observed in type 2 diabetic patients and in animal models of diabetes. Thus, it is fascinating that originally developed as an efficient strategy for the lowering of circulating cholesterol levels, BAS have secondary actions that extend beyond their effects plasma cholesterol levels.

PHYSICAL ACTIVITY AND THE METABOLIC SYNDROME

Abundant evidence gathered from epidemiological, prospective and intervention studies suggest that MetS is a condition associated with physical inactivity. It is not surprising that physical activity is associated with a reduced cardiovascular risk. Moreover, a rapidly progressing body of human and animal data confirms an important beneficial role for physical activity for a number of metabolic derangements associated with MetS (Figure 3) and thus physical activity constitutes an essential part in managing those derangements. For example, exercise training has been shown to reduce body weight and visceral fat accumulation [64-67], improve insulin sensitivity [64-66], increase high-density lipoprotein cholesterol and decrease triglyceride levels [67, 68], as well as decreasing blood pressure [67, 69].

Altogether, physical activity has mild to moderate effects on the metabolic and cardiovascular risk factors entailed in MetS. In fact, individuals who engage in regular moderate or intensive physical activity are half as likely to develop MetS as sedentary individuals [14]. Importantly, a physically active lifestyle does not only prevent MetS but also acts beneficial in its treatment, by beneficially modulating its risk factors. Hereby regular physical exercise also decreased complications associated with MetS like type 2 diabetes [70, 71], atherosclerosis [72-75], cardiovascular events [76] and premature mortality [77]. Thus, adopting and maintaining a physically

active lifestyle is highly beneficial in the prevention and treatment of the MetS by modulation of its risk factors.

Current recommendations for the prevention and treatment of MetS are based on evidence from epidemiological and intervention studies. Herein, the US Center for Disease Control and Prevention (CDC), the American College of Sports Medicine (ACSM) and the US Surgeon General have recommended that adults engage in at least 30 min of moderate-intensity physical activity on most, and preferably all, days of the week [67, 78]. Most studies investigating the effect of physical activity on MetS and its individual risk factors have focused on vigorous endurance training, but the benefits of brisk walking and lifestyle activities are becoming increasingly recognized.

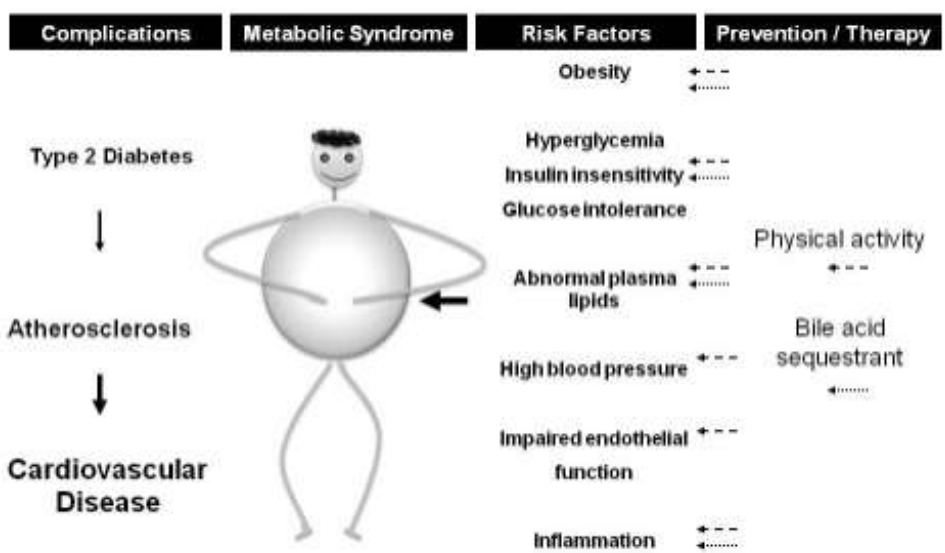


Figure 3. The effects of physical activity or bile acid sequestration on risk factors of the Metabolic Syndrome. A dashed arrow with stripes denotes a beneficial effect of physical activity while an arrow dashed with points denotes a beneficial effect of bile acid sequestration on a specific risk factor. No arrow is equivalent to currently unknown. The risk factors hyperglycemia, insulin insensitivity and glucose intolerance are depicted jointly to denote major problems in glucose homeostasis associated with MetS risk.

Physical activity and cholesterol homeostasis

Physical activity has shown to not only effect total cholesterol and lipoprotein levels favorably, but also those of triglycerides. This is evident from acute and chronic physical activity interventions. For example, a single bout of physical activity decreased plasma triglycerides and increased plasma high density lipoprotein (HDL) cholesterol [79]. Increases in plasma HDL have also been observed upon chronic physical activity, along with decreases in plasma triglycerides and the atherogenic low density lipoprotein (LDL) cholesterol [68, 80-84].

Although physical activity imposes a rather small effect (about 3-5%) on decreasing LDL and increasing HDL cholesterol [82], this small effect of exercise on dyslipidemia should not be underestimated. As stated earlier, HDL is an important player in RCT and an increase in HDL potentially increases cholesterol excretion from the body *via* RCT. Surprisingly the concept of an enhanced RCT induced by physical activity has never been explored fully. However, few and limited studies indicate that RCT might be enhanced upon physical activity. For example athletes have shown a greater capacity for cholesterol efflux from fibroblasts [85] as well as mouse macrophage cells [86] compared to sedentary controls. This is intriguing, because the beneficial effects of physical activity on various MetS risk factors (for further reading refer to BOX 2) and cardiovascular disease have been widely documented but the mechanisms behind the beneficial effects of physical activity remain elusive.

Specifically, little is known about the effects of physical activity on the turnover of cholesterol into bile acids, and its effects on bile acid metabolism per se. Limited past work indicates that exercise potentially modulates cholesterol and bile acid metabolism [87-90]. For example, an increased fecal bile acid and neutral sterol production has been observed in middle aged long distance runners [87]. Furthermore, early studies on female rats showed that six weeks of voluntary wheel running promoted an increased biliary bile acid, cholesterol and phospholipids secretion [89] and either increased or had no effect on bile flow [89, 90]. A recent study in mice also showed that twelve weeks of forced treadmill exercise prevents gallstone formation in gallstone-prone mice [88]. Gallstone disease is

characterized by an abnormally high biliary cholesterol to bile acid and phospholipids ratio [91]. Granted from these previous observations, it is likely that exercise enhances pathways leading to increased fecal sterol excretion and that these pathways might play an important role in the beneficial effects of physical activity on MetS risk factors and MetS induced complications.

SCOPE OF THIS THESIS

MetS can, over years, lead to type 2 diabetes, atherosclerosis and life-threatening cardiovascular events. The dauntingly increasing prevalence and economic burden of MetS throughout the world warrants efficient treatment strategies. Herein, BAS represent an attractive pharmacological interjection, while physical activity represents an attractive lifestyle intervention. Both strategies display pleiotrophic effects on a variety of complications associated with MetS; however, underlying mechanisms are not understood. In this thesis the effect of BAS treatment and physical activity was tested on specific metabolic imbalances associated with MetS. We elucidated the effects of BAS treatment or physical activity on parameters of cholesterol and glucose homeostasis. Thereby the work presented in this thesis provides novel mechanistic insights on how BAS treatment and physical activity bring about their beneficial effects in the MetS and its associated complications.

Originally developed as cholesterol lowering agents, BAS have recently emerged as agents that improve glucose homeostasis and insulin sensitivity. However, the underlying mechanisms are largely unknown. We speculated that BAS-induced changes in hepatic lipid and glucose metabolism are underlying mediators herein. In **Chapter 2**, we first evaluated how BAS affects bile acid and lipid metabolism. First, we show that BAS surprisingly did not alter the bile acid pool size in diabetic mice despite a massive fecal loss. Second, we show that BAS induced *de novo* fatty acid synthesis in the livers of lean and diabetic mice, while plasma glucose levels decreased upon BAS in diabetic mice. Subsequently, we

investigated whether specific modifications in hepatic glucose homeostasis mediate the BAS-induced reduction in blood glucose in diabetic mice **(Chapter 3)**. We found that BAS modulated blood glucose levels by increasing the metabolic clearance rate of glucose in diabetic mice. Concomitantly, BAS-treatment decreased skeletal muscle acylcarnitine contents, which have been associated with insulin resistance. In another set of studies we then evaluated whether physical activity decreases MetS risk by modulating cholesterol homeostasis. Here, we first investigated the effect of voluntary wheel running on parameters of cholesterol and bile acid metabolism in healthy chow-fed mice (**Chapter 4)** and show that voluntary wheel running increased whole-body cholesterol turnover by stimulating bile acid synthesis and decreasing cholesterol absorption. To our surprise, we observed in a next study **(Chapter 5)** that *in vivo* macrophage-to-feces RCT was not increased in healthy chow-fed mice exposed to a voluntary running wheel. However, exposing hypercholesterolemic, atherogenic mice to the voluntary wheel running intervention led to a reduced atherosclerotic lesion size development which coincided with an increased bile acid synthesis, biliary secretion and fecal excretion **(Chapter 6)**. Lastly, we tested the effects of BAS on atherosclerotic lesion size development and cholesterol homeostasis in hypercholesterolemic, atherogenic mice **(Chapter 7)**. Next to the dramatic improvements in plasma lipids and lipoprotein profiles, we found a ~78% reduction in atherosclerotic lesion size development which was accompanied by a switch from cholesterol accumulation to cholesterol loss upon BAS treatment.

REFERENCES

1 Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J. and Flegal, K. M., Prevalence of Overweight and Obesity in the United States, 1999-2004, JAMA, 2006, 295: 1549-1555.

2 Ogden, C. L., Carroll, M. D. and Flegal, K. M., High Body Mass Index for Age Among US Children and Adolescents, 2003-2006, JAMA, 2008, 299: 2401-2405.

3 Wang, Y. and Beydoun, M. A., The Obesity Epidemic in the United States Gender, Age, Socioeconomic, Racial/Ethnic, and Geographic Characteristics: A Systematic Review and Meta-Regression Analysis, Epidemiol Rev, 2007, 29: 6-28.

4 Allcock, D. M., Gardner, M. J. and Sowers, J. R., Relation between Childhood Obesity and Adult Cardiovascular Risk, Int J Pediatr Endocrinol, 2009, 2009: 108187.

5 Haslam, D. W. and James, W. P. T., Obesity, The Lancet, 2005, 366: 1197-1209.

6 Sinha, R., Fisch, G., Teague, B., Tamborlane, W. V., Banyas, B., Allen, K., Savoye, M., Rieger, V., Taksali, S., Barbetta, G., Sherwin, R. S. and Caprio, S., Prevalence

of impaired glucose tolerance among children and adolescents with marked obesity, N Engl J Med, 2002, 346: 802-810.
7 Daniels, S. R. M. D. P., Arnett, D. K. P., Eckel, R. H. M. D., Gidding, S. S. M. D., Hayman, L. L. P. R. N., Kumanyika, S. P. M. P. H. R. D., Robinson, T. N. M. D. M. P. H., Scott, B. J. R. D. M. P. H., St. Jeor, S. P. and Williams, C. L. M. D. M. P. H., Overweight in Children and Adolescents: Pathophysiology, Consequences, Prevention, and Treatment, Circulation, 2005, 111: 1999-2012.
8 Kohen-Avramoglu, R., Theriault, A. and Adeli, K., Emergence of the metabolic syndrome in childhood: an epidemiological overview and mechanistic link to dyslipidemia, Clinical Biochemistry, 2003, 36: 413-420.
9 Ford, E. S., Prevalence of the Metabolic Syndrome Defined by the International Diabetes Federation Among Adults in the U.S, Diabetes Care, 2005, 28: 2745-2749.
10 Maumus, S., Marie, B. r. r., Siest, G. r. and Visvikis-Siest, S., A Prospective Study on the Prevalence of Metabolic Syndrome Among Healthy French Families, Diabetes Care, 2005, 28: 675-682.
11 Balkau, B., Vernay, M., Mhamdi, L., Novak, M., Arondel, D., Vol, S., Tichet, J. and Eschwege, E., The incidence and persistence of the NCEP (National Cholesterol Education Program) metabolic syndrome. The French D.E.S.I.R. study, Diabetes Metab, 2003, 29: 526-532.
12 Liese, A. D., Mayer-Davis, E. J. and Haffner, S. M., Development of the multiple metabolic syndrome: an epidemiologic perspective, Epidemiol Rev, 1998, 20: 157-172.
13 Lakka, H. M., Laaksonen, D. E., Lakka, T. A., Niskanen, L. K., Kumpusalo, E., Tuomilehto, J. and Salonen, J. T., The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men, JAMA, 2002, 288: 2709-2716.
14 Laaksonen, D. E., Lakka, H. M., Salonen, J. T., Niskanen, L. K., Rauramaa, R. and Lakka, T. A., Low levels of leisure-time physical activity and cardiorespiratory fitness predict development of the metabolic syndrome, Diabetes Care, 2002, 25: 1612-1618.
15 Laaksonen, D. E., Niskanen, L., Lakka, H. M., Lakka, T. A. and Uusitupa, M., Epidemiology and treatment of the metabolic syndrome, Ann Med, 2004, 36: 332-346.
16 Ardern, C. I. and Janssen, I., Metabolic syndrome and its association with morbidity and mortality, Appl Physiol Nutr Metab, 2007, 32: 33-45.
17 Alberti, K. G. and Zimmet, P. Z., Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation, Diabet Med, 1998, 15: 539-553.
18 Moyna, N. M., Robertson, R. J., Meckes, C. L., Peoples, J. A., Millich, N. B. and Thompson, P. D., Intermodal comparison of energy expenditure at exercise intensities corresponding to the perceptual preference range, Med Sci Sports Exerc, 2001, 33: 1404-1410.
19 Expert Panel on Detection, E. and Treatment of High Blood Cholesterol in Adults, Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), JAMA, 2001, 285: 2486-2497.
20 Grundy, S. M., Brewer, H. B., Jr, Cleeman, J. I., Smith, S. C., Jr, Lenfant, C. and for the Conference Participants, Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition, Circulation, 2004, 109: 433-438.
21 Lakka, H.-M., Laaksonen, D. E., Lakka, T. A., Niskanen, L. K., Kumpusalo, E., Tuomilehto, J. and Salonen, J. T., The Metabolic Syndrome and Total and Cardiovascular Disease Mortality in Middle-aged Men, JAMA, 2002, 288: 2709-2716.
22 Klein, B. E. K., Klein, R. and Lee, K. E., Components of the Metabolic Syndrome and Risk of Cardiovascular Disease and Diabetes in Beaver Dam, Diabetes Care, 2002, 25: 1790-1794.

23 Haffner, S. M., Lehto, S., Ronnemaa, T., Pyorala, K. and Laakso, M., Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction, N Engl J Med, 1998, 339: 229-234.
24 Pyorala, K., Laakso, M. and Uusitupa, M., Diabetes and atherosclerosis: an epidemiologic view, Diabetes Metab Rev, 1987, 3: 463-524.
25 Tribble, D. L., Holl, L. G., Wood, P. D. and Krauss, R. M., Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size, Atherosclerosis, 1992, 93: 189-199.
26 Vergès, B., Lipid modification in type 2 diabetes: the role of LDL and HDL, Fundamental & Clinical Pharmacology, 2009, 23: 681-685.
27 Chapman, M. J., Guerin, M. and Bruckert, E., Atherogenic, dense low-density lipoproteins. Pathophysiology and new therapeutic approaches, Eur Heart J, 1998, 19 Suppl A: A24-30.
28 Sviridov, D., Mukhamedova, N., T. Remaley, A., Chin-Dusting, J. and Nestel, P., Antiatherogenic Functionality of High Density Lipoprotein: How Much versus How Gooden-subtitle=, Journal of Atherosclerosis and Thrombosis, 2008, 15: 52-62.
29 Fielding, C. J. and Fielding, P. E., Molecular physiology of reverse cholesterol transport, J Lipid Res, 1995, 36: 211-228.
30 Kashyap, M. L., Basic considerations in the reversal of atherosclerosis: significance of high-density lipoprotein in stimulating reverse cholesterol transport, Am J Cardiol, 1989, 63: 56H-59H.
31 Diagnosis and classification of diabetes mellitus, Diabetes Care, 2004, 27 Suppl 1: S5-S10.
32 Hutley, L. P. and Prins, J. B. M. B. P., Fat as an Endocrine Organ: Relationship to the Metabolic Syndrome, American Journal of the Medical Sciences, 2005, 330: 280-289.
33 Kuipers, F., Stroeve, J. H., Caron, S. and Staels, B., Bile acids, farnesoid X receptor, atherosclerosis and metabolic control, Curr Opin Lipidol, 2007, 18: 289-297.
34 Kuipers, F. a., Stroeve, J. H. M. a., Caron, S. b. and Staels, B. b., Bile acids, farnesoid X receptor, atherosclerosis and metabolic control, Current Opinion in Lipidology, 2007, 18: 289-297.
35 Kobayashi, M., Ikegami, H., Fujisawa, T., Nojima, K., Kawabata, Y., Noso, S., Babaya, N., Itoi-Babaya, M., Yamaji, K., Hiromine, Y., Shibata, M. and Ogihara, T., Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin, Diabetes, 2007, 56: 239-247.
36 The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease, JAMA, 1984, 251: 351-364.
37 The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering, JAMA, 1984, 251: 365-374.
38 Brensike, J. F., Levy, R. I., Kelsey, S. F., Passamani, E. R., Richardson, J. M., Loh, I. K., Stone, N. J., Aldrich, R. F., Battaglini, J. W., Moriarty, D. J. and et al., Effects of therapy with cholestyramine on progression of coronary arteriosclerosis: results of the NHLBI Type II Coronary Intervention Study, Circulation, 1984, 69: 313-324.
39 Blankenhorn, D. H., Nessim, S. A., Johnson, R. L., Sanmarco, M. E., Azen, S. P. and Cashin-Hemphill, L., Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts, JAMA, 1987, 257: 3233-3240.
40 Cashin-Hemphill, L., Mack, W. J., Pogoda, J. M., Sanmarco, M. E., Azen, S. P. and Blankenhorn, D. H., Beneficial effects of colestipol-niacin on coronary atherosclerosis. A 4-year follow-up, JAMA, 1990, 264: 3013-3017.
41 Kane, J. P., Malloy, M. J., Ports, T. A., Phillips, N. R., Diehl, J. C. and Havel, R. J., Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens, JAMA, 1990, 264: 3007-3012.

42 Watts, G. F., Lewis, B., Brunt, J. N., Lewis, E. S., Coltart, D. J., Smith, L. D., Mann, J. I. and Swan, A. V., Effects on coronary artery disease of lipid-lowering diet, or diet plus cholestyramine, in the St Thomas' Atherosclerosis Regression Study (STARS), Lancet, 1992, 339: 563-569.
43 Aldridge, M. A. and Ito, M. K., Colesevelam hydrochloride: a novel bile acid-binding resin, Ann Pharmacother, 2001, 35: 898-907.
44 Yamaoka-Tojo, M., Tojo, T. and Izumi, T., Beyond cholesterol lowering: pleiotropic effects of bile acid binding resins against cardiovascular disease risk factors in patients with metabolic syndrome, Curr Vasc Pharmacol, 2008, 6: 271-281.
45 Isaacsohn, J., Hunninghake, D., Schrott, H., Dujovne, C. A., Knopp, R., Weiss, S. R., Bays, H., Crouse, J. R., 3rd, Davidson, M. H., Keilson, L. M., McKenney, J., Korenman, S. G., Dobs, A. S., Stein, E., Krauss, R. M., Maccubbin, D., Cho, M., Plotkin, D. J. and Mitchel, Y. B., Effects of simvastatin, an HMG-CoA reductase inhibitor, in patients with hypertriglyceridemia, Clin Cardiol, 2003, 26: 18-24.
46 Staels, B. and Kuipers, F., Bile acid sequestrants and the treatment of type 2 diabetes mellitus, Drugs, 2007, 67: 1383-1392.
47 Zhang, Y., Lee, F. Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F. J., Willson, T. M. and Edwards, P. A., Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice, Proc Natl Acad Sci U S A, 2006, 103: 1006-1011.
48 Biddinger, S. B., Haas, J. T., Yu, B. B., Bezy, O., Jing, E., Zhang, W., Unterman, T. G., Carey, M. C. and Kahn, C. R., Hepatic insulin resistance directly promotes formation of cholesterol gallstones, Nat Med, 2008, 14: 778-782.
49 Hyogo, H., Roy, S., Paigen, B. and Cohen, D. E., Leptin promotes biliary cholesterol elimination during weight loss in ob/ob mice by regulating the enterohepatic circulation of bile salts, J Biol Chem, 2002, 277: 34117-34124.
50 Twisk, J., Hoekman, M. F., Lehmann, E. M., Meijer, P., Mager, W. H. and Princen, H. M., Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase gene transcription, Hepatology, 1995, 21: 501-510.
51 Suzuki, T., Oba, K., Futami, S., Suzuki, K., Ouchi, M., Igari, Y., Matsumura, N., Watanabe, K., Kigawa, Y. and Nakano, H., Blood glucose-lowering activity of colestimide in patients with type 2 diabetes and hypercholesterolemia: a case-control study comparing colestimide with acarbose, J Nippon Med Sch, 2006, 73: 277-284.
52 Garg, A. and Grundy, S. M., Cholestyramine therapy for dyslipidemia in non-insulin-dependent diabetes mellitus. A short-term, double-blind, crossover trial, Ann Intern Med, 1994, 121: 416-422.
53 Zieve, F. J., Kalin, M. F., Schwartz, S. L., Jones, M. R. and Bailey, W. L., Results of the glucose-lowering effect of WelChol study (GLOWS): a randomized, double-blind, placebo-controlled pilot study evaluating the effect of colesevelam hydrochloride on glycemic control in subjects with type 2 diabetes, Clin Ther, 2007, 29: 74-83.
54 Bays, H. E., Goldberg, R. B., Truitt, K. E. and Jones, M. R., Colesevelam hydrochloride therapy in patients with type 2 diabetes mellitus treated with metformin: glucose and lipid effects, Arch Intern Med, 2008, 168: 1975-1983.
55 Fonseca, V. A., Rosenstock, J., Wang, A. C., Truitt, K. E. and Jones, M. R., Colesevelam HCl improves glycemic control and reduces LDL cholesterol in patients with inadequately controlled type 2 diabetes on sulfonylurea-based therapy, Diabetes Care, 2008, 31: 1479-1484.
56 Goldberg, R. B., Fonseca, V. A., Truitt, K. E. and Jones, M. R., Efficacy and safety of colesevelam in patients with type 2 diabetes mellitus and inadequate glycemic control receiving insulin-based therapy, Arch Intern Med, 2008, 168: 1531-1540.
57 Kobayashi, M., Ikegami, H., Fujisawa, T., Nojima, K., Kawabata, Y., Noso, S., Babaya, N., Itoi-Babaya, M., Yamaji, K., Hiromine, Y., Shibata, M. and Ogihara, T.,

Prevention and Treatment of Obesity, Insulin Resistance, and Diabetes by Bile Acidâ€“Binding Resin, Diabetes, 2007, 56: 239-247.
58 Lefebvre, P., Cariou, B., Lien, F., Kuipers, F. and Staels, B., Role of Bile Acids and Bile Acid Receptors in Metabolic Regulation, Physiol. Rev., 2009, 89: 147-191.
59 Ma, K., Saha, P. K., Chan, L. and Moore, D. D., Farnesoid X receptor is essential for normal glucose homeostasis, In, Journal of Clinical Investigation, American Society for Clinical Investigation, 2006: 1102-1109.
60 Shang, Q., Saumoy, M., Holst, J. J., Salen, G. and Xu, G., Colesevelam improves insulin resistance in a diet-induced obesity (F-DIO) rat model by increasing the release of GLP-1, Am J Physiol Gastrointest Liver Physiol, 298: G419-424.
61 Koike, K., Murakami, K., Nozaki, N., Sugiura, K. and Inoue, M., Colestilan, a new bile acid-sequestering resin, reduces bodyweight in postmenopausal women who have dieted unsuccessfully, Drugs R D, 2005, 6: 273-279.
62 Bays, H. E., Davidson, M., Jones, M. R. and Abby, S. L., Effects of colesevelam hydrochloride on low-density lipoprotein cholesterol and high-sensitivity C-reactive protein when added to statins in patients with hypercholesterolemia, Am J Cardiol, 2006, 97: 1198-1205.
63 Devaraj, S., Autret, B. and Jialal, I., Effects of colesevelam hydrochloride (WelChol) on biomarkers of inflammation in patients with mild hypercholesterolemia, Am J Cardiol, 2006, 98: 641-643.
64 Ivy, J. L., Role of exercise training in the prevention and treatment of insulin resistance and non-insulin-dependent diabetes mellitus, Sports Med, 1997, 24: 321-336.
65 Ross, R., Dagnone, D., Jones, P. J., Smith, H., Paddags, A., Hudson, R. and Janssen, I., Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men. A randomized, controlled trial, Ann Intern Med, 2000, 133: 92-103.
66 Rice, B., Janssen, I., Hudson, R. and Ross, R., Effects of aerobic or resistance exercise and/or diet on glucose tolerance and plasma insulin levels in obese men, Diabetes Care, 1999, 22: 684-691.
67 Physical Activity and Health: A Report of the Surgeon General, In: U. S. Department of Health and Human Services, C. f. D. C. a. P., National Center for Chronic Disease Prevention and Health Promotion (ed), Atlanta, GA, 1996.
68 Haskell, W. L., The influence of exercise on the concentrations of triglyceride and cholesterol in human plasma, Exerc Sport Sci Rev, 1984, 12: 205-244.
69 Arroll, B. and Beaglehole, R., Does physical activity lower blood pressure: a critical review of the clinical trials, J Clin Epidemiol, 1992, 45: 439-447.
70 Helmrich, S. P., Ragland, D. R., Leung, R. W. and Paffenbarger, R. S., Jr., Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus, N Engl J Med, 1991, 325: 147-152.
71 Laaksonen, D. E., Lindstrom, J., Lakka, T. A., Eriksson, J. G., Niskanen, L., Wikstrom, K., Aunola, S., Keinanen-Kiukaanniemi, S., Laakso, M., Valle, T. T., Ilanne-Parikka, P., Louheranta, A., Hamalainen, H., Rastas, M., Salminen, V., Cepaitis, Z., Hakumaki, M., Kaikkonen, H., Harkonen, P., Sundvall, J., Tuomilehto, J. and Uusitupa, M., Physical activity in the prevention of type 2 diabetes: the Finnish diabetes prevention study, Diabetes, 2005, 54: 158-165.
72 Lakka, T. A., Venalainen, J. M., Rauramaa, R., Salonen, R., Tuomilehto, J. and Salonen, J. T., Relation of leisure-time physical activity and cardiorespiratory fitness to the risk of acute myocardial infarction, N Engl J Med, 1994, 330: 1549-1554.
73 Manson, J. E., Hu, F. B., Rich-Edwards, J. W., Colditz, G. A., Stampfer, M. J., Willett, W. C., Speizer, F. E. and Hennekens, C. H., A prospective study of walking as compared with vigorous exercise in the prevention of coronary heart disease in women, N Engl J Med, 1999, 341: 650-658.

74 Sesso, H. D., Paffenbarger, R. S., Jr. and Lee, I. M., Physical activity and coronary heart disease in men: The Harvard Alumni Health Study, Circulation, 2000, 102: 975-980.
75 Tanasescu, M., Leitzmann, M. F., Rimm, E. B., Willett, W. C., Stampfer, M. J. and Hu, F. B., Exercise type and intensity in relation to coronary heart disease in men, JAMA, 2002, 288: 1994-2000.
76 Lakka, T. A. and Laaksonen, D. E., Physical activity in prevention and treatment of the metabolic syndrome, Appl Physiol Nutr Metab, 2007, 32: 76-88.
77 Katzmarzyk, P. T., Leon, A. S., Wilmore, J. H., Skinner, J. S., Rao, D. C., Rankinen, T. and Bouchard, C., Targeting the metabolic syndrome with exercise: evidence from the HERITAGE Family Study, Med Sci Sports Exerc, 2003, 35: 1703-1709.
78 Pate, R. R., Pratt, M., Blair, S. N., Haskell, W. L., Macera, C. A., Bouchard, C., Buchner, D., Ettinger, W., Heath, G. W., King, A. C. and et al., Physical activity and public health. A recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine, JAMA, 1995, 273: 402-407.
79 Thompson, P. D., Crouse, S. F., Goodpaster, B., Kelley, D., Moyna, N. and Pescatello, L., The acute versus the chronic response to exercise, Med Sci Sports Exerc, 2001, 33: S438-445; discussion S452-433.
80 Huttunen, J. K., Lansimies, E., Voutilainen, E., Ehnholm, C., Hietanen, E., Penttila, I., Siitonen, O. and Rauramaa, R., Effect of moderate physical exercise on serum lipoproteins. A controlled clinical trial with special reference to serum high-density lipoproteins, Circulation, 1979, 60: 1220-1229.
81 Kraus, W. E., Houmard, J. A., Duscha, B. D., Knetzger, K. J., Wharton, M. B., McCartney, J. S., Bales, C. W., Henes, S., Samsa, G. P., Otvos, J. D., Kulkarni, K. R. and Slentz, C. A., Effects of the amount and intensity of exercise on plasma lipoproteins, N Engl J Med, 2002, 347: 1483-1492.
82 Leon, A. S. and Sanchez, O. A., Response of blood lipids to exercise training alone or combined with dietary intervention, Med Sci Sports Exerc, 2001, 33: S502-515; discussion S528-509.
83 Stefanick, M. L., Mackey, S., Sheehan, M., Ellsworth, N., Haskell, W. L. and Wood, P. D., Effects of diet and exercise in men and postmenopausal women with low levels of HDL cholesterol and high levels of LDL cholesterol, N Engl J Med, 1998, 339: 12-20.
84 Williams, P. T., Krauss, R. M., Stefanick, M. L., Vranizan, K. M. and Wood, P. D., Effects of low-fat diet, calorie restriction, and running on lipoprotein subfraction concentrations in moderately overweight men, Metabolism, 1994, 43: 655-663.
85 Gupta, A. K., Ross, E. A., Myers, J. N. and Kashyap, M. L., Increased reverse cholesterol transport in athletes, Metabolism, 1993, 42: 684-690.
86 Wei, C., Penumetcha, M., Santanam, N., Liu, Y. G., Garelnabi, M. and Parthasarathy, S., Exercise might favor reverse cholesterol transport and lipoprotein clearance: potential mechanism for its anti-atherosclerotic effects, Biochim Biophys Acta, 2005, 1723: 124-127.
87 Sutherland WH, N. E., Macfarlane DJ, Williamson SA, Robertson MC., Cholesterol metabolism n distance runners., Clin Physiol., 1992, 12: 29-37.
88 Wilund, K. R., Feeney, L. A., Tomayko, E. J., Chung, H. R. and Kim, K., Endurance exercise training reduces gallstone development in mice, J Appl Physiol, 2008, 104: 761-765.
89 Yiamouyiannis, C. A., Martin, B. J. and Watkins, J. B., 3rd, Chronic physical activity alters hepatobiliary excretory function in rats, J Pharmacol Exp Ther, 1993, 265: 321-327.
90 Watkins, J. B., 3rd, Crawford, S. T. and Sanders, R. A., Chronic voluntary exercise may alter hepatobiliary clearance of endogenous and exogenous chemicals in rats, Drug Metab Dispos, 1994, 22: 537-543.
91 Marschall, H. U. and Einarsson, C., Gallstone disease, J Intern Med, 2007, 261: 529-542.

92 Bjorntorp, P., Visceral obesity: a "civilization syndrome", Obes Res, 1993, 1: 206-222.
93 Bjorntorp, P., Abdominal obesity and the metabolic syndrome, Ann Med, 1992, 24: 465-468.
94 Björntorp, P., Body fat distribution, insulin resistance, and metabolic diseases, Nutrition, 1997, 13: 795-803.
95 Pasquali, R., Vicennati, V., Cacciari, M. and Pagotto, U., The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome, Ann N Y Acad Sci, 2006, 1083: 111-128.
96 Kotronen, A., Yki-Jarvinen, H., Sevastianova, K., Bergholm, R., Hakkarainen, A., Pietilainen, K. H., Juurinen, L., Lundbom, N. and Sorensen, T. I., Comparison of the Relative Contributions of Intra-Abdominal and Liver Fat to Components of the Metabolic Syndrome, Obesity (Silver Spring).
97 Gimbrone, M. A., Jr., Topper, J. N., Nagel, T., Anderson, K. R. and Garcia-Cardena, G., Endothelial dysfunction, hemodynamic forces, and atherogenesis, Ann N Y Acad Sci, 2000, 902: 230-239; discussion 239-240.
98 Huang, P. L., Unraveling the links between diabetes, obesity, and cardiovascular disease, Circ Res, 2005, 96: 1129-1131.
99 Kim, J. A., Montagnani, M., Koh, K. K. and Quon, M. J., Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms, Circulation, 2006, 113: 1888-1904.
100 Coakley, E. H., Rimm, E. B., Colditz, G., Kawachi, I. and Willett, W., Predictors of weight change in men: results from the Health Professionals Follow-up Study, Int J Obes Relat Metab Disord, 1998, 22: 89-96.
101 Erlichman, J., Kerbey, A. L. and James, W. P., Physical activity and its impact on health outcomes. Paper 2: Prevention of unhealthy weight gain and obesity by physical activity: an analysis of the evidence, Obes Rev, 2002, 3: 273-287.
102 Hu, F. B., Li, T. Y., Colditz, G. A., Willett, W. C. and Manson, J. E., Television watching and other sedentary behaviors in relation to risk of obesity and type 2 diabetes mellitus in women, JAMA, 2003, 289: 1785-1791.
103 Jeffery, R. W., McGuire, M. T. and French, S. A., Prevalence and correlates of large weight gains and losses, Int J Obes Relat Metab Disord, 2002, 26: 969-972.
104 Saris, W. H., Blair, S. N., van Baak, M. A., Eaton, S. B., Davies, P. S., Di Pietro, L., Fogelholm, M., Rissanen, A., Schoeller, D., Swinburn, B., Tremblay, A., Westerterp, K. R. and Wyatt, H., How much physical activity is enough to prevent unhealthy weight gain? Outcome of the IASO 1st Stock Conference and consensus statement, Obes Rev, 2003, 4: 101-114.
105 Irwin, M. L., Yasui, Y., Ulrich, C. M., Bowen, D., Rudolph, R. E., Schwartz, R. S., Yukawa, M., Aiello, E., Potter, J. D. and McTiernan, A., Effect of exercise on total and intra-abdominal body fat in postmenopausal women: a randomized controlled trial, JAMA, 2003, 289: 323-330.
106 Jakicic, J. M., Marcus, B. H., Gallagher, K. I., Napolitano, M. and Lang, W., Effect of exercise duration and intensity on weight loss in overweight, sedentary women: a randomized trial, JAMA, 2003, 290: 1323-1330.
107 Slentz, C. A., Duscha, B. D., Johnson, J. L., Ketchum, K., Aiken, L. B., Samsa, G. P., Houmard, J. A., Bales, C. W. and Kraus, W. E., Effects of the amount of exercise on body weight, body composition, and measures of central obesity: STRRIDE--a randomized controlled study, Arch Intern Med, 2004, 164: 31-39.
108 Lee, S., Kuk, J. L., Katzmarzyk, P. T., Blair, S. N., Church, T. S. and Ross, R., Cardiorespiratory fitness attenuates metabolic risk independent of abdominal subcutaneous and visceral fat in men, Diabetes Care, 2005, 28: 895-901.

109 Henriksen, E. J., Invited review: Effects of acute exercise and exercise training on insulin resistance, J Appl Physiol, 2002, 93: 788-796.
110 Holloszy, J. O., Exercise-induced increase in muscle insulin sensitivity, J Appl Physiol, 2005, 99: 338-343.
111 Ross, R., Does exercise without weight loss improve insulin sensitivity?, Diabetes Care, 2003, 26: 944-945.
112 Kenney, M. J. and Seals, D. R., Postexercise hypotension. Key features, mechanisms, and clinical significance, Hypertension, 1993, 22: 653-664.
113 Dickinson, H. O., Mason, J. M., Nicolson, D. J., Campbell, F., Beyer, F. R., Cook, J. V., Williams, B. and Ford, G. A., Lifestyle interventions to reduce raised blood pressure: a systematic review of randomized controlled trials, J Hypertens, 2006, 24: 215-233.
114 Whelton, S. P., Chin, A., Xin, X. and He, J., Effect of aerobic exercise on blood pressure: a meta-analysis of randomized, controlled trials, Ann Intern Med, 2002, 136: 493-503.
115 Pescatello, L. S., Franklin, B. A., Fagard, R., Farquhar, W. B., Kelley, G. A. and Ray, C. A., American College of Sports Medicine position stand. Exercise and hypertension, Med Sci Sports Exerc, 2004, 36: 533-553.
116 Vider, J., Laaksonen, D. E., Kilk, A., Atalay, M., Lehtmaa, J., Zilmer, M. and Sen, C. K., Physical exercise induces activation of NF-kappaB in human peripheral blood lymphocytes, Antioxid Redox Signal, 2001, 3: 1131-1137.
117 Kasapis, C. and Thompson, P. D., The effects of physical activity on serum C-reactive protein and inflammatory markers: a systematic review, J Am Coll Cardiol, 2005, 45: 1563-1569.
118 Sen, C. K. and Packer, L., Antioxidant and redox regulation of gene transcription, FASEB J, 1996, 10: 709-720.
119 Church, T. S., Barlow, C. E., Earnest, C. P., Kampert, J. B., Priest, E. L. and Blair, S. N., Associations between cardiorespiratory fitness and C-reactive protein in men, Arterioscler Thromb Vasc Biol, 2002, 22: 1869-1876.
120 Lakka, T. A., Laaksonen, D. E., Lakka, H. M., Mannikko, N., Niskanen, L. K., Rauramaa, R. and Salonen, J. T., Sedentary lifestyle, poor cardiorespiratory fitness, and the metabolic syndrome, Med Sci Sports Exerc, 2003, 35: 1279-1286.
121 Lakka, T. A., Lakka, H. M., Rankinen, T., Leon, A. S., Rao, D. C., Skinner, J. S., Wilmore, J. H. and Bouchard, C., Effect of exercise training on plasma levels of C-reactive protein in healthy adults: the HERITAGE Family Study, Eur Heart J, 2005, 26: 2018-2025.
122 Mattusch, F., Dufaux, B., Heine, O., Mertens, I. and Rost, R., Reduction of the plasma concentration of C-reactive protein following nine months of endurance training, Int J Sports Med, 2000, 21: 21-24.
123 Tisi, P. V., Hulse, M., Chulakadabba, A., Gosling, P. and Shearman, C. P., Exercise training for intermittent claudication: does it adversely affect biochemical markers of the exercise-induced inflammatory response?, Eur J Vasc Endovasc Surg, 1997, 14: 344-350.
124 You, T., Berman, D. M., Ryan, A. S. and Nicklas, B. J., Effects of hypocaloric diet and exercise training on inflammation and adipocyte lipolysis in obese postmenopausal women, J Clin Endocrinol Metab, 2004, 89: 1739-1746.
125 Obisesan, T. O., Leeuwenburgh, C., Phillips, T., Ferrell, R. E., Phares, D. A., Prior, S. J. and Hagberg, J. M., C-reactive protein genotypes affect baseline, but not exercise training-induced changes, in C-reactive protein levels, Arterioscler Thromb Vasc Biol, 2004, 24: 1874-1879.
126 Rauramaa, R., Halonen, P., Vaisanen, S. B., Lakka, T. A., Schmidt-Trucksass, A., Berg, A., Penttila, I. M., Rankinen, T. and Bouchard, C., Effects of aerobic physical exercise on inflammation and atherosclerosis in men: the DNASCO Study: a six-year randomized, controlled trial, Ann Intern Med, 2004, 140: 1007-1014.

127 Powers, S. K. and Hamilton, K., Antioxidants and exercise, Clin Sports Med, 1999, 18: 525-536.
128 Hansson, G. K., Inflammation, atherosclerosis, and coronary artery disease, N Engl J Med, 2005, 352: 1685-1695.
129 Green, D. J., Maiorana, A., O'Driscoll, G. and Taylor, R., Effect of exercise training on endothelium-derived nitric oxide function in humans, J Physiol, 2004, 561: 1-25.
130 Hambrecht, R., Wolf, A., Gielen, S., Linke, A., Hofer, J., Erbs, S., Schoene, N. and Schuler, G., Effect of exercise on coronary endothelial function in patients with coronary artery disease, N Engl J Med, 2000, 342: 454-460.
131 Harris, M. B., Mitchell, B. M., Sood, S. G., Webb, R. C. and Venema, R. C., Increased nitric oxide synthase activity and Hsp90 association in skeletal muscle following chronic exercise, Eur J Appl Physiol, 2008, 104: 795-802.
132 Fung Ping, L., Lai Ming, Y., Laher, I., Xiaoqiang, Y., Zhen Yu, C. and Yu, H., Exercise, Vascular Wall and Cardiovascular Diseases, In, Sports Medicine, ADIS International Limited, 2008: 1006-1024.

SUPPLEMENTAL

BOX 1

Metabolic syndrome, type 2 diabetes and atherosclerosis

The central features of MetS comprise visceral obesity, insulin resistance, hyperglycemia, atherogenic dyslipidemia, hypertension and endothelial dysfunction. Of these the first two seem more critical in the development of MetS than the others, as other risk factors are associated with obesity and insulin resistance. For example, weight loss can lead to an improvement of various metabolic risk factors simultaneously. Thus, adiposity is, to a certain degree, required for the manifestation of MetS. However, there are also individuals who are obese but who do not display any of the other metabolic derangements that comprise MetS. Therefore, both insulin resistance and a metabolic disposition for obesity appear to be required for the expression of MetS. Endothelial dysfunction is a consequence of both insulin resistance and visceral adipose tissue. Thus, the constellation of metabolic derangements comprising MetS are interrelated, share common mediators, pathways and pathophysiological mechanisms. This represents a vicious cycle, where one risk factor predisposes to another.

Visceral obesity

Central abdominal obesity, but not general obesity, has consistently been shown to represent a strong risk factor for cardiovascular disease in prospective population studies [92]. Further, strong correlations between the waist / hip ratio, the waist circumference, but not the body mass index, and a clustering of MetS risk factors [93] have demonstrated that central obesity plays a key factor in MetS. Enlarged visceral adipose tissue contribute to an increased free fatty acid production and release of pro-inflammatory adipokines and cytokines, which all impair insulin-mediated glucose uptake and promote insulin resistance [94]. Moreover, it has been suggested that visceral obesity may derive from chronic maladaptation to environmental stress factors [95]. Herein, the individual's inability to cope with stress is thought to increase hypothalamus-pituitary-axis (HPA) activity and activation of the sympathetic nervous system which can bring about visceral obesity. In fact, a dysregulation of the HPA-axis has consistently been demonstrated upon visceral obesity [95]. In addition to visceral fat depots, fatty liver has recently also been shown to explain variations in plasma triglyceride, glucose, HDL, cholesterol, insulin, and hepatic insulin sensitivity and thus been suggested to be an important predictor of MetS [96].

Insulin Resistance

Insulin is an anabolic hormone that is produced by the pancreas in response to high plasma glucose levels and stimulates glucose utilization by various tissues and thereby the clearance of glucose from the circulation. In addition, insulin also effects vascular function and promotes vasodilation and blood flow to target tissues for an efficient glucose clearance. The target tissues of peripheral glucose disposal are the liver, skeletal muscle and the adipose tissue. Through intricate signaling pathways, insulin promotes peripheral glucose uptake by translocating specific glucose transporters to the cell surface; GLUT 4 (SLC2A4) in skeletal muscle and adipose tissue and GLUT2 (SLC2A2) in liver. Insulin clearly promotes glucose storage over breakdown as demonstrated by insulin's actions to stimulate glycogen synthesis in the liver and skeletal muscle and to inhibit glycogenolysis

(breakdown of glycogen) but also the production of new glucose in the liver (gluconeogenesis). Hereby insulin thus prevents an increase of glucose flux towards the blood. Additionally, insulin prevents fat break down (lipolysis) but stimulates glucose uptake in fat tissues. Insulin resistance is characterized by high circulating plasma glucose levels at prevailing (high) insulin concentrations due to an inability of liver, skeletal muscle and adipose tissue to respond to insulin. Specific impairments in the cellular insulin signaling pathways interfere with the translocation of the glucose transporter to the cell surface in insulin resistance leading to an impaired glucose uptake. Insulin resistance moreover constitutes a condition where signaling pathways that promote vasodilation are impaired while processes that stimulate endothelial dysfunction are up-regulated, thus predisposing to atherosclerosis. Hyperglycemia, toxicity from free fatty acids, obesity, dyslipidemia and pro-inflammatory conditions, all contributors to insulin resistance can also affect vascular function.

Metabolic syndrome and endothelial dysfunction

Endothelial dysfunction is a consequence of many cardiovascular risk factors, leads to hypertension and has major contributions in the development of atherosclerosis [97-99]. Endothelial cells line the inner surface of blood vessels. They act and respond to physiological and pathological stimuli and produce vasoactive substances for proper endothelial function. Visceral adiposity and insulin resistance give rise to increased levels of circulating glucose, free fatty acids, pro-inflammatory cytokines, adipokines and oxidative stress, all substances that promote endothelial damage eventually leading to endothelial dysfunction.

BOX 2: PHYSICAL ACTIVITY AND MetS RISK FACTORS NOT DISCUSSED IN THIS THESIS

Physical activity and obesity

Epidemiological studies have shown that sedentary behaviors such as watching TV, playing video games and computer-related work promote unhealthy weight gain and obesity while regular physical activity prevents them [100-104]. Some studies

indicate that in order to decrease adiposity, the amount of physical activity is more important than its intensity [65, 105-107]. It is currently not understood whether exercise preferentially decreases visceral or subcutaneous abdominal fat [65, 105, 107, 108]. However, it has been shown that exercise training decreases total, visceral and subcutaneous abdominal fat even without weight loss in normal weight, obese, abdominal obese and type 2 diabetic individuals [108].

Physical activity and insulin sensitivity

Insulin sensitivity improves by physical activity in an acute and chronic manner. The acute benefits of physical activity on insulin sensitivity are mediated by exercise-induced modulations of the insulin signaling response to muscle contraction. Herein, an exercise-induced increase in insulin-dependent glucose transporter 4 (GLUT4) translocation to the muscle cell surface has been demonstrated [109, 110]. Moreover, it was found that this particular effect lasts for only 48–72 h post exercise, which strengthens the general recommendation to maintain a daily exercise regime in order to maximize the benefits of exercise on insulin sensitivity.

Clearly, exercise training also has beneficial effects on reducing obesity, which in itself constitutes an important mechanism for the improvements in insulin sensitivity. The differentiation between the independent effects of exercise on obesity and insulin sensitivity proofs difficult [111].

Nevertheless, chronic exercise interventions have shown to result in specific skeletal muscle adaptations including increased GLUT4 content, glycogen synthase activity, mitochondrial enzyme activity, capillary and mitochondrial density, improvements in endothelial function and potentially switching to a more oxidative fiber type [109, 110]. All these favorable modulations induced by physical activity in skeletal muscle tissue are indicative of an improved glucose uptake and contribute to the chronic effects of exercise on improving insulin sensitivity.

Physical activity and hypertension

During a bout of physical activity systolic blood pressure increases relative to the physical effort. Intriguingly, 2-4 hours after at least 20 min of low and moderate physical activity systolic blood pressure decreases below the resting level, about 8-10 mmHg in normo-tensive individuals, while the decrease can be 16-20 mmHg in hypertensive individuals [79, 112]. Meta-analysis of randomized controlled trials revealed that the chronic effect of exercise training yields a decrease of about 3.8 mmHg in systolic and 2.6 mmHg in diastolic blood pressure in normo-tensives and a slightly stronger reduction in hypertensive individuals [113, 114]. It is currently believed that exercise decreases blood pressure by its beneficial effects on body composition, insulin sensitivity, endothelial function and autonomic nervous system balance [115].

Physical activity and inflammation

It is known that a single bout of exercise induces an acute inflammatory response, as demonstrated by an increased release of pro-inflammatory cytokines and increased plasma concentrations of C-reactive protein [116, 117], while also increasing oxidative stress [118]. From a chronic viewpoint, however, lower plasma levels of inflammatory markers have consistently been observed in individuals with high cardio-respiratory fitness compared to those with low cardio-respiratory fitness [117, 119, 120]. Yet, data on the effects of physical activity on inflammatory markers in individuals at low to moderate cardiovascular risk are conflicting, and while some report beneficial effects [121-125] others report no effect of physical activity on inflammatory markers [126]. Despite the apparent discrepancies between studies, regular physical activity and maintenance of cardio-respiratory fitness may reduce low-grade inflammation contrary to the acute inflammatory response of a single bout of exercise. The proposed mechanisms mediating the exercise-induced reduction of inflammation are associated with reductions in body fat and associated production of pro-inflammatory cytokines, improved dyslipidemia as well as endothelial function, up-regulated cellular defenses, i.e., increased

antioxidant enzymes, an increased production of anti-inflammatory cytokines as well as a reduced expression of adhesion molecules [67, 117, 127-129].

Physical activity and endothelial function

Obesity, hypertension, type 2 diabetes and hypercholesterolemia are all risk factors of the MetS and are all associated with impaired endothelial function. Physical activity has consistently shown to improve endothelial function, as measured *via* flow-mediated dilation of the brachial artery in obese, hypertensive, diabetic and hypercholesterolemic individuals [129, 130]. Additionally, physical activity has shown to improve endothelial function as demonstrated by an enhanced endothelial nitric oxide synthase activity [131, 132]. Enhancing endothelial nitric oxide activity leads to an increased production of the potent vasodilator nitric oxide.

CHAPTER 2

Bile acid sequestration induces hepatic *de novo* lipogenesis via FXR and LXRα-controlled metabolic pathways in mice

H. Herrema, M. Meissner, T.H. van Dijk, G. Brufau, R. Boverhof, M.H. Oosterveer, D-J. Reijngoud, M. Müller, F. Stellaard,, A.K. Groen,
F. Kuipers

Hepatology. 2010 Mar;51(3):806-16

ABSTRACT

Diabetes is characterized by high blood glucose levels and dyslipidemia. Bile acid sequestration has been found to improve both plasma glycaemic control and cholesterol profiles in diabetic patients. Yet, bile acid sequestration is also known to affect triglyceride metabolism, possibly via signaling pathways involving FXR and LXRα. We quantitatively assessed kinetic parameters of bile acid metabolism in lean C57Bl/6 and in obese, diabetic *db/db* mice upon bile acid sequestration using colesevelam HCl (2% wt/wt in diet) and related these to quantitative changes in hepatic lipid metabolism. As expected, bile acid sequestration reduced intestinal bile acid reabsorption. Importantly, bile acid pool size and biliary bile acid secretion remained unchanged upon sequestrant-treatment due to compensation by *de novo* bile acid synthesis in both models. Nevertheless, lean and *db/db* mice showed increased, mainly periportally confined, hepatic triglyceride contents, increased expression of lipogenic genes and increased fractional contributions of newly synthesized fatty acids. Lipogenic gene expression was not induced in sequestrant-treated $Fxr^{-/-}$ and $Lxr\alpha^{-/-}$ mice compared to wild-type littermates, in line with reports indicating a regulatory role of FXR and LXRα in bile acid-mediated regulation of hepatic lipid metabolism. Conclusion: Bile acid sequestration by colesevelam induces the lipogenic pathway in an FXR- and LXRα-dependent manner without affecting the total pool size of bile acid in mice. We speculate that a shift from intestinal reabsorption to *de novo* synthesis as source of bile acid upon bile acid sequestration affects zonation of metabolic processes within the liver acinus.

INTRODUCTION

Diabetes is a multifactorial disease characterized by increased fasting blood glucose levels and dyslipidemia *i.e.*, high plasma triglyceride and low-density-lipoprotein cholesterol (LDL-C) levels. Controlling blood glucose and cholesterol levels in diabetic patients is critical for delaying the progression of clinical complications *e.g.*, neuropathy and cardiovascular diseases. An efficient way to reduce plasma cholesterol levels is to induce cholesterol secretion in bile, either as bile acid or as free cholesterol. Bile is secreted into the ileum to facilitate absorption of lipids and lipid soluble vitamins. About 95% of secreted bile acids are reabsorbed in the terminal ileum and transported back to the liver via the portal vein (enterohepatic circulation). Besides their function in absorption of dietary fats, bile acids are signaling molecules and exert an important role in regulation of lipid metabolism[1]. Interestingly, bile acid metabolism is affected in diabetes which might contribute to the altered lipid profile observed in these patients[2]. Knowledge of potential disturbances in bile acid metabolism in type 2 diabetic humans and animal models, however, is still very limited[3].

Increasing fecal bile acid loss by preventing their intestinal reabsorption (sequestration) increases bile acid synthesis and, hence, hepatic cholesterol turnover. As a consequence, LDL-C levels are reduced in hyperlipidemic subjects[4, 5]. Interestingly, bile acid sequestration also improves glucose levels in type 2 diabetic patients[6-8]. Yet, use of bile acid sequestrants has been associated with elevated plasma triglyceride levels[9, 10]. Bile acid feeding on the other hand has been shown to improve plasma lipid profiles in these patients[11, 12]. The regulation of the interrelationship between bile acid and lipid metabolism is still only partly understood. At a molecular level, a key regulatory role is assigned to the bile acid-activated nuclear receptor FXR (NR1H4)[13]. Pharmacological activation of FXR has been shown to improve hypertriglyceridemia in mouse models of insulin resistance[14, 15] whereas *Fxr*$^{-/-}$ mice have increased serum triglyceride levels[16]. Moreover, administration of the natural FXR-ligand cholate improved plasma triglyceride levels of high fat diet-fed mice via SHP-dependent modulation of the lipogenic gene *Srebp1c*[17]. In the same study, it was shown that the nuclear

oxysterol receptor LXRα (NR1H3) is involved in the regulation of lipogenic gene expression upon bile acid feeding.

At a physiological level, bile acid-activated signaling pathways are regulated by bile acid concentrations in the liver. We hypothesized that an altered flux of bile acids returning to the liver underlies, at least in part, the consequences on hepatic metabolism observed upon bile acid sequestration. We quantitatively assessed the kinetics of bile acid and hepatic fatty acid metabolism in lean C57Bl/6 mice and in obese and diabetic *db/db* mice treated with the bile acid sequestrant colesevelam HCl[18]. Additionally, we studied the contribution of FXR and LXRα to sequestrant-induced changes in lipogenic gene expression.

Bile acid sequestration reduced intestinal reabsorption of bile acids by 30%. Nevertheless, the bile acid pool size remained unchanged in both models due to a compensatory increase in *de novo* synthesis of bile acids. Remarkably, sequestrant-treatment significantly increased hepatic triglyceride contents which primarily accumulated in periportal areas. Expression levels of lipogenic genes as well as the fractional contribution of de novo synthesized fatty acids were increased. This lipogenic response appeared to be FXR- and LXRα-dependent. We speculate that a shift from reabsorption to *de novo* synthesis as the source of biliary bile acids underlies the lipogenic phenotype observed upon bile acid sequestration.

METHODS AND MATERIASL

Animals

Male lean C57Bl/6 and obese, diabetic *db/db* mice on a C57Bl/6 background (B6.Cg-*m* +/+ *Lepr*^{db}/J) were purchased from Charles River Laboratories (L'Arbresle, France and Brussels, Belgium, respectively). *Fxr*$^{-/-}$- [19] and *Lxrα*$^{-/-}$ mice[20] were generated as described. All animals were housed individually in a temperature- and light-controlled facility. Mice were fed commercially available laboratory chow (RMH-B; Arie Blok, Woerden, The Netherlands), when indicated supplemented with 2% (w/w) colesevelam HCl (Daiichi Sankyo, Inc., Parsippany,

NJ, USA), for 2 weeks. Mice were used for experimental procedures at 12 weeks of age. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Experimental procedures

Postprandial blood glucose levels were measured at the start of the experiment and subsequently after 1 week and after 2 weeks of treatment. Additionally, body weight and food intake were determined at these time points. [1-^{13}C]-acetate (2% w/v in drinking water) was provided for 24 h (7AM-7AM), starting at day 13 of the experiment. Blood spots were collected from the tail on filter paper (Schleicher and Schuell No2992, 's Hertogenbosch, The Netherlands) before and after administration of the label. Blood spots were air-dried and stored at room temperature until analysis. After two weeks on the diets, mice were sacrificed by heart puncture under isoflurane anesthesia. Plasma was stored at -20°C until analyzed. The liver was removed, weighed and snap-frozen in liquid nitrogen. The intestine was excised, flushed with cold (4°C) PBS and subsequently snap-frozen in liquid nitrogen. Both liver and intestine were stored at -80ºC until biochemical analysis and RNA isolation.

In a separate experiment, 400 µg [$^{2}H_4$]-cholate (in 0.5% NaHCO in PBS, pH = 7.4) was intravenously administered at day ten of the two-week periods. Subsequently, retro-orbital blood samples (75 µl) were obtained at 12, 24, 36, 48 and 60 h after injection of [$^{2}H_4$]-cholate in chow-fed lean and *db/db* mice. A pilot study in colesevelam-treated animals indicated that, as expected, turnover of [$^{2}H_4$]-cholate was markedly increased. Therefore, blood samples were obtained at 12, 18, 24, 30 and 36 h after administration of [$^{2}H_4$]-cholate in colesevelam-treated lean and *db/db* mice. Plasma was stored at -20°C until analyzed. Feces were collected over the 60 h experimental period and, after air-drying, kept at room temperature until analysis. After 60 h, mice were anesthetized by intraperitoneal injection of Hypnorm (1 ml/kg) and Diazepam (10 mg/kg) and subjected to gallbladder cannulation for 30 min. During bile collection, body temperature was stabilized using a humidified incubator. Bile was stored at -20°C until analyzed. Animals were sacrificed by cervical dislocation.

Analytical procedures

Blood glucose concentrations were measured using EuroFlashTM test strips (LifeScan Benelux, Beerse, Belgium). Hepatic lipids were extracted according to Bligh & Dyer[21]. Plasma and liver triglyceride and cholesterol contents were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma free fatty acids were determined using a NEFA-C kit (Waco Chemicals, Neuss, Germany). Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium). Triglycerides in each fraction were determined. Total bile acids in bile and feces were determined by an enzymatic fluorimetric assay[22].Liver morphology was assessed by Masson's trichrome staining of parafin embedded material.

GC/MS analysis and mass isotopomer distribution analysis (MIDA)

Biliary and fecal bile acids were determined by gas chromatography as described [23]. The isotope dilution technique as well as the preparation of plasma samples for analysis of bile acids by gas chromatography-mass spectrometry (GC-MS) were described in detail by Hulzebos *et al*[23]. Fecal neutral sterols were analyzed as described[24]. Labeling of acetyl-CoA pools with orally provided [1-^{13}C]-acetate was described by Jung *et al*[25]. Cholesterol was extracted from blood spots and prepared for GC-MS analysis as described[26]. Lipids in liver homogenates were hydrolyzed in HCl/acetonitril. Fatty acids were extracted in hexane and converted to their pentafluorobenzyl (PFB) derivatives. The fatty acid-PFB isotopomer patterns (mass fragments C16:0 m/z 255-259, C18:0 m/z 283-287, C18:1 m/z 281-285) were analyzed using a Agilent 5975 series GC-MS (Agilent Technologies, Santa Clara, CA). GC-MS measurements of fatty acids and MIDA analyses were performed essentially as described[27, 28].

Calculation of *de novo* synthesis and chain elongation of fatty acids

Incorporation of [1-^{13}C]-acetate into C16:0 was assumed to be solely the result of *de novo* lipogenesis via the malonyl-CoA-Fas pathway. The M1 and M3 mass

isotopomers of C16:0 were used to calculate the acetyl CoA precursor pool and fractional synthesis of C16:0 as described[28].

C18:0 is synthesized from chain elongation of pre-existing (chain elongation) and *de novo* synthesized C16:0 (*de novo* lipogenesis). The M1 mass isotopomer of C18:0 represents the sum of these two processes; the M3 mass isotopomer results only from chain elongation of *de novo* synthesized C16:0. We assumed that the acetate precursor pool enrichment calculated for C16:0 equalled the precursor pool of acetate used to elongate C16:0. C18:0 generated from *de novo* synthesized C16:0 was considered as nonamer of acetate. Therefore, MIDA algorithms using M3 of C18:0 and the acetate precursor pool enrichment calculated for C16:0 were used to calculate the fractional C18:0 synthesis. Subsequently, total M1 mass isotopomer of C18:0 was corrected for the contribution of C18:0 derived from *de novo* synthesized C16:0. Using this methodology, we could calculate the contribution of chain elongation of pre-existing C16:0 to the total fractional C18:0 synthesis.

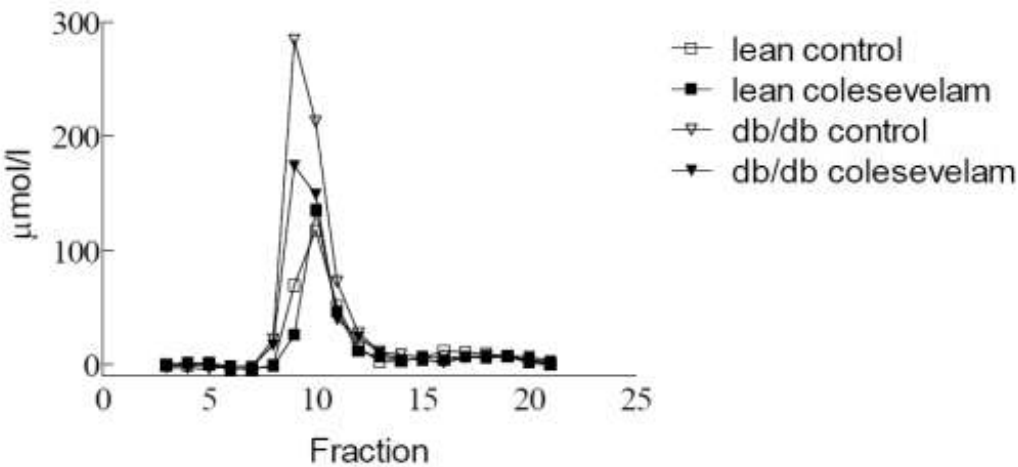
C18:1 is synthesized by desaturation of C18:0 by Scd1. Using similar methodology to that of C18:0, we could calculate the contribution of chain elongation of pre-existing C16:0 and *de novo* synthesized C16:0 to total fractional synthesis of C18:1.

Throughout the manuscript, chain elongation is defined as synthesis of C18:0 or C18:1 from pre-existing C16:0; *de novo* lipogenesis is defined as the synthesis of C18:0 or C18:1 from *de novo* synthesized C16:0.

Calculation of CDCA- and CA-derived bile acids

The murine bile acid species we quantified in bile and feces using GC-MS include cholate, deoxycholate, chenodeoxycholate, α-muricholate, β-muricholate, ω-muricholate, hyodeoxycholate and ursodeoxycholate. We consider cholate and deoxycholate as CA-derived and the others as CDCA-derived bile acids. The contribution of deoxycholate to CA-derived bile acids is minor in bile (~5%), because the majority of DCA that returns to the liver is reconverted to CA in mice. Therefore, the pool size and synthesis rate of cholate as determined *in vivo* using [$^2H_4$]-cholate were used as values for CA-derived bile acid pool size and synthesis.

The composition and synthesis of CDCA-derived bile acids were calculated from the ratio of CDCA-derived/CA-derived bile acids and the CA-derived pool and synthesis as determined by calculations of [$^2H_4$]-cholate. The product of the CDCA/CA ratio in feces and CA synthesis represents the synthesis of CDCA-derived bile acids. The product of the CDCA/CA ratio in bile and CA pool size represents the contribution of CDCA-derived bile acids to the total bile acid pool size.



Supplemental Figure 1. Plasma lipoprotein profile of control and colesevelam-treated lean and db/db mice. VLDL-TG was reduced in diabetic mice upon treatment. No changes in VLDL-TG levels were observed in lean mice. Plasma from 6 animals per group was pooled and separated by fast protein liquid chromatography (FPLC) on a Superose 6 column.

RNA isolation

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described[29]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to 18S (liver) and β-actin (intestine).

Statistics

All values are represented as mean ± standard deviation. Statistical analysis was assessed using the Mann-Whitney-U-test (SPSS 12.0.1 for Windows). P-values were corrected for multiple comparison errors.

RESULTS

Effects of colesevelam-treatment on food intake, body weight and plasma metabolites in lean and *db/db* mice

Lean and *db/db* mice were treated with the bile acid sequestrant colesevelam for two weeks. Food intake was increased in colesevelam-treated lean and *db/db* mice during treatment compared to untreated controls (Table 1). Body weight gain was unaffected in colesevelam-treated lean mice but decreased in colesevelam-treated *db/db* mice. Blood glucose levels increased in control *db/db* mice during the two-week intervention period but remained stable in colesevelam-treated *db/db* mice. Blood glucose levels remained unchanged upon treatment in lean mice. Fasting insulin levels (not shown) were unchanged and decreased, respectively, in colesevelam-treated lean and *db/db* mice. Non-esterified fatty acid (NEFA) and VLDL-triglyceride levels (Supplemental Figure 1) were significantly reduced in colesevelam-treated *db/db* mice compared to untreated controls but remained unchanged in lean mice.

Biliary bile acid flux and cholate pool size remain unchanged in colesevelam-treated lean and *db/db* mice

Control *db/db* mice showed increased feces production and a higher fecal bile acid output, representing hepatic bile acid synthesis, compared to lean controls (Figure 1A and B, resp.). As expected, colesevelam-treatment led to massive increases in fecal bile acid output (Figure 1B). Untreated lean and *db/db* mice had similar bile flow rates and biliary bile acid output rates (Figure 1C and D, resp.) which remained unchanged in both models upon sequestrant-treatment.

Table 1. Basal, plasma and liver parameters of lean and *db/db* mice (control and colesevelam-treated). Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.

	lean control	lean colesevelam	*db/db* control	*db/db* colesevelam
Basal parameters				
Cumulative weight gain (g)	1.0 ± 0.3	0.7 ± 0.5	1.4 ± 0.6	-1.0 ± 1.3 #
Cumulative food intake(g)	31.7 ± 11.8	57.1 ± 8.4 #	60.7 ± 8.7*	79.2 ± 4.0 *#
24h food intake (g)	2.4 ± 0.9	4.4 ± 0.6#	4.7 ± 0.7 *	6.1 ± 0.3*#
24h water intake (ml)	7.5 ±1.6	7.1 ± 0.5	18.3 ± 3.6	17.1 ±1.7 #
Glucose T=0 (mmol/l)	8.9 ±1.2	8.9 ±0.4	22.6 ± 6.4 *	22.1 ± 3.6 *
Glucose T=1 week (mmol/l)	8.8 ± 1.2	8.9 ± 1.1	26.2 ± 7.2 *	24.0 ± 5.8 *
Glucose T=2 weeks (mmol/l)	8.7 ± 0.6	8.5 ± 0.3	30.4 ± 3.7 *	22.9 ± 2.5 *#
Plasma				
Triglycerides (mmol/l)	1.07 ± 0.45	1.15 ± 0.22	2.83 ± 0.92 *	1.98 ± 0.68*#
Cholesterol (mmol/l)	2.01 ± 0.27	1.96 ± 0.12	3.49 ± 0.23 *	3.38 ± 0.52 *
NEFA (umol/l)	190 ± 47	197 ± 35	540 ± 183 *	428 ± 170 *#
Liver				
Liver (% BW)	5.0 ± 0.6	5.3 ± 0.2	4.1 ± 0.3	5.2 ± 1.0
Triglycerides (µmol/g)	10.4 ± 1.7	15.9 ± 2.4 *	39.7 ± 9.6 *	51.7 ± 8.1 *#
Cholesterol (µmol /g)	4.0 ± 0.7	4.7 ± 1.3	4.9 ± 2.8	5.3 ± 1.7
Phospholipids (µmol /g)	20.2 ± 1.2	18.9 ± 3.9	19.5 ± 3.0	20.3 ± 4.9

Direct end-products of *de novo* bile acid synthesis are the primary bile acids cholate and chenodeoxycholate. Modifications of these bile acids in liver and intestine give rise to differentially structured primary and secondary bile acids, respectively. Supplemental Table 1 provides details on biliary and fecal bile acid compositions. In short, sequestrant-treatment resulted in a strongly increased relative content of fecal deoxycholate in both groups. Cholate remained the major biliary bile acid species in both models upon sequestrant-treatment. Next, we determined relevant kinetic parameters of cholate[23], the major primary bile acid species in mice. Untreated *db/db* mice displayed a larger pool size and a higher synthesis rate of cholate compared to untreated lean mice (Figure 2). Importantly, cholate pool size remained unchanged upon colesevelam-treatment in both models. Synthesis rates of cholate were massively increased upon sequestrant-

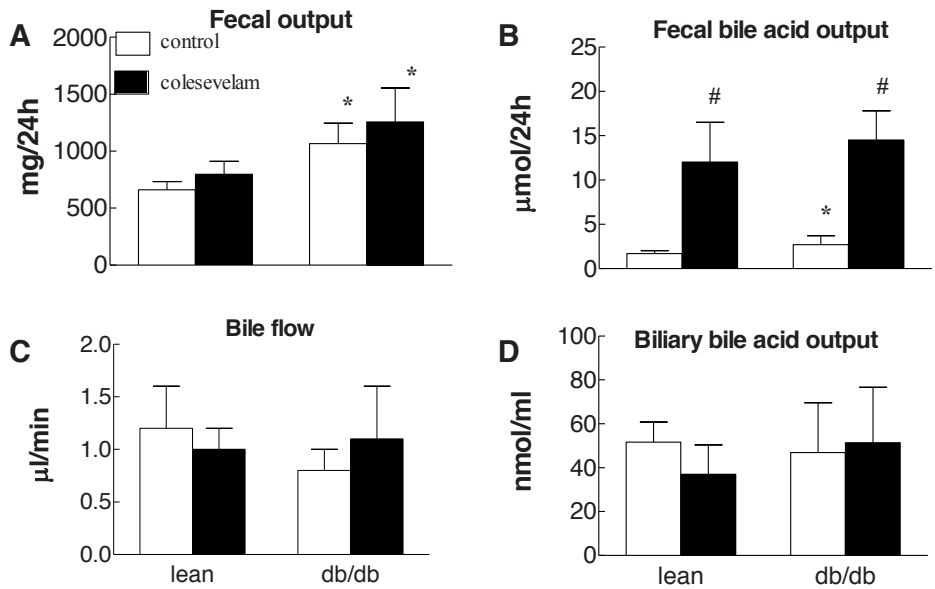


Figure 1. Effects of colesevelam on fecal and biliary bile acids. Fecal output (A) was unchanged in both lean and *db/db* mice upon colesevelam-treatment. As expected, fecal bile acid output (B) of lean and *db/db* mice was increased upon colesevelam-treatment, indicative for higher hepatic bile acid synthesis. Importantly, bile flow (C) and biliary bile acid output (D) were unchanged upon colesevelam treatment. Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.

treatment (+375% and +172%, lean and *db/db* mice, respectively) and completely compensated for the increased fecal bile acid loss induced by colesevelam. The calculated amount of cholate reabsorbed from intestines of colesevelam-treated lean and *db/db* mice was reduced by about 30% compared to untreated controls (Figure 2D). Decreased plasma bile acid levels further reflect a reduced flux of bile acids returning to the liver (Figure 2E).

To gain insight into colesevelam-induced changes in total bile acid pool composition and synthesis of bile acids derived from the primary bile acid species cholate and chenodeoxycholate, we calculated the amount of cholate- and chenodeoxycholate-derived bile acids in the pool as well as their synthesis rates. Upon sequestrant-treatment, the total pools of bile acids remained unchanged in both models (Figure 3A). Nevertheless, the pool size of chenodeoxycholate-derived bile acids was decreased. The synthesis of cholate-derived bile acids was

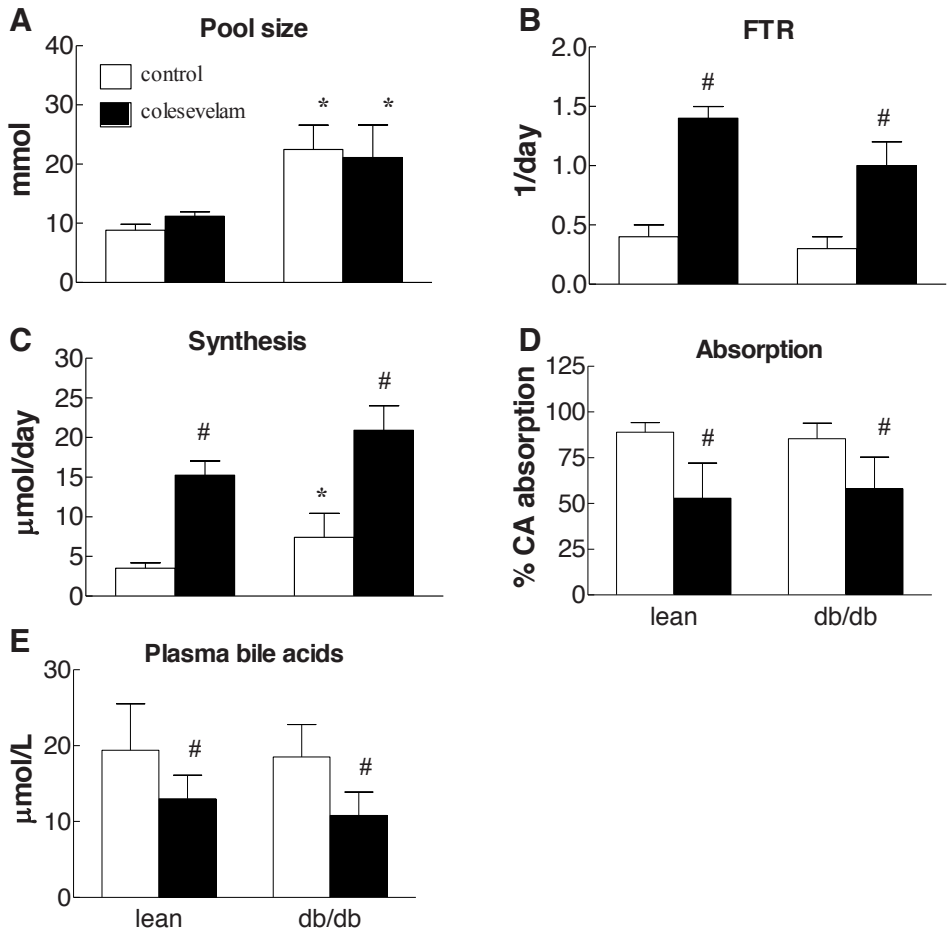


Figure 2. The pool size of cholate is unchanged in lean and *db/db* mice after a two-week colesevelam-treatment. The kinetics of cholate were calculated from the decay of [$^2H_4$]-cholate from plasma (18). Despite substantial fecal bile acid loss (Figure 1B) in colesevelam-treated lean and *db/db* mice, cholate pool size (A) was unchanged in both groups. Hence, the fractional turnover rate (FTR) (B) and synthesis rate (C) of cholate were massively increased. Both lean and *db/db* mice had reduced absorption of cholate (D) upon treatment with colesevelam. Additionally, total plasma bile acids (E) were reduced in colesevelam-treated mice. Reabsorbed cholate is represented as percent of cholate secreted in bile. Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.

massively increased, whereas synthesis of chenodeoxycholate-derived bile acids remained unchanged in sequestrant-treated mice compared to untreated controls (Figure 3B). Corresponding with increased cholate synthesis, hepatic expression of Cyp7a1, encoding the first and rate-limiting enzyme in bile acid synthesis, and

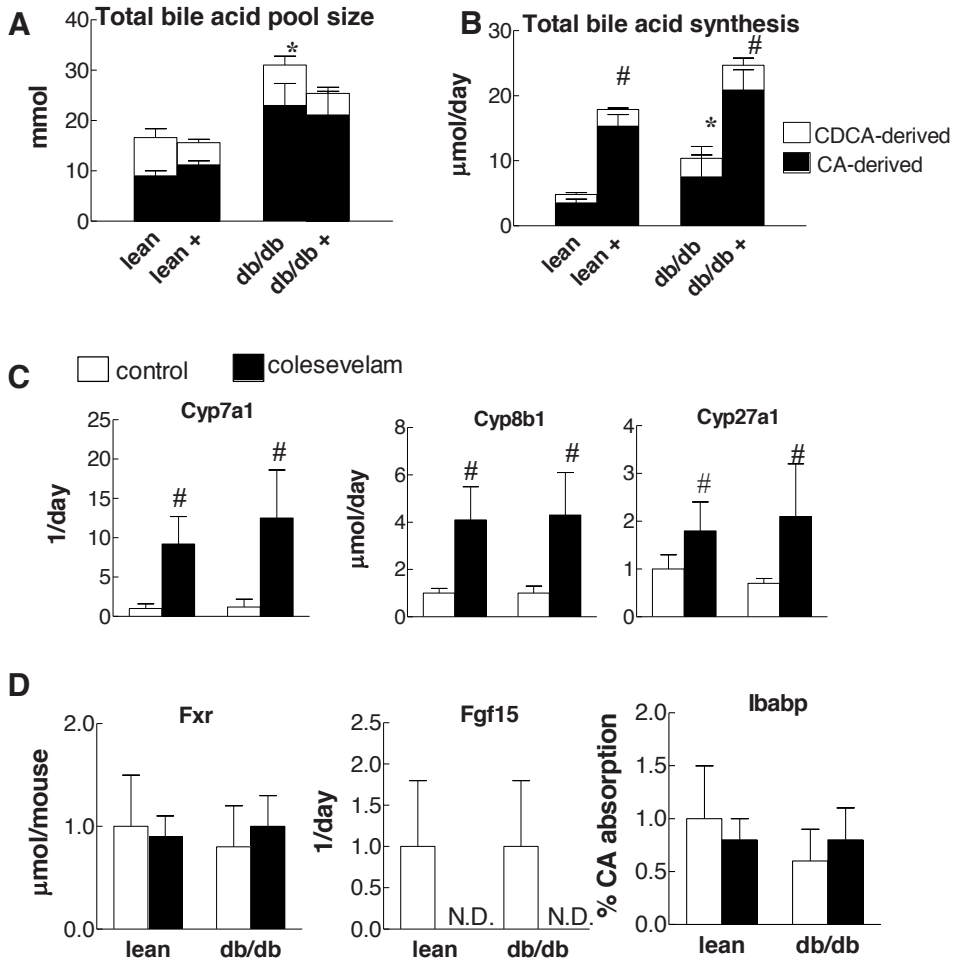


Figure 3. Decreased chenodeoxycholate (CDCA)-derived bile acid pool size and synthesis in colesevelam-treated mice. The composition and synthesis of the total bile acid pool were calculated from the ratio of CDCA-derived/cholate (CA)-derived bile acids and the pool size and synthesis of cholate as determined in vivo using [$^2H_4$]-cholate. The product of CDCA-derived/CA-derived bile acid ratio in feces and CA synthesis represents the synthesis of CDCA-derived bile acids. The product of the CDCA-derived/CA-derived bile acid ratio in bile and CA pool size represents the contribution of CDCA-derived bile acids to the total bile acid pool size. The total bile acid pool size (A) was unchanged in colesevelam-treated lean and *db/db* mice. Synthesis (B) of CA-derived bile acids was massively increased whereas synthesis of CDCA-derived bile acids was unaffected. Hepatic expression (C) of Cyp7a1, Cyp8b1 and Cyp27a1 were massively increased. Interestingly, intestinal expression (D) of the FXR-target gene Fgf15 was undetectable in colesevelam-treated mice whereas expression of Ibabp (Fabp6), another FXR target gene was unchanged. Values are presented as mean ± SD. In panel A-B, * and # indicate significant differences in total (CA + CDCA-derived) pool and synthesis. Gene expression levels are presented as relative expression normalized to 36B4 (liver) and 18S (intestine). N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively. +; colesevelam-treated. CA-derived; cholate + deoxycholate. CDCA-derived;

chenodeoxycholate, α-muricholate, β-muricholate, ώ-muricholate, hyodeoxycholate and ursodeoxycholate.

Cyp8b1, encoding the specific enzyme required for cholate synthesis, were massively increased. Cyp27a1 directs bile acid synthesis towards CDCA. Although expression of Cyp27a1 was increased in colesevelam-treated mice, this was not reflected in increased CDCA synthesis (Figure 3C). Despite the fact that bile acid reabsorption was not completely abolished, expression levels of the Fxr-target gene Fgf15 were undetectable in distal ilea of colesevelam-treated lean and *db/db* mice (Figure 3D).

Cholesterol synthesis is massively increased in colesevelam-treated lean and *db/db* mice colesevelam-treatment increased fecal cholesterol excretion (Figure 4A). Together with a strongly increased synthesis of bile acids this translates into an increased turnover of cholesterol. However, this did not result in reduced plasma concentrations or hepatic contents of cholesterol (Figure 4B and C, respectively). Increased hepatic expression of Hmgr, encoding the rate-controlling enzyme in cholesterol synthesis, and of *Ldlr* (Figure 4D) indicated the anticipated hepatic compensatory response in cholesterol metabolism after colesevelam-treatment. To quantify this, the fraction of newly synthesized cholesterol was determined by analysis of the incorporation of [1-^{13}C]-acetate into plasma cholesterol. Fractional cholesterol synthesis was indeed robustly increased in colesevelam-treated mice (Figure 4E).

Hepatic triglyceride content is increased due to enhanced contribution of lipogenesis in colesevelam-treated mice

Both colesevelam-treated lean and *db/db* mice had modestly increased (lean +50%, *db/db* +23%) hepatic triglyceride contents compared to untreated controls (Table 1). Remarkably, fat accumulated primarily in periportal areas upon bile acid sequestration (Figure 5A and B). Increased hepatic expression of key lipogenic genes (Srebp1c, Acc1, Fasn and Scd1) (Figure 5C) was highly suggestive of enhanced synthesis of fatty acids. Indeed, the total fractions of newly synthesized C16:0, C18:0 and C18:1, as determined by incorporation of [1-^{13}C]-acetate followed by MIDA analysis, confirmed that synthesis of these major hepatic fatty

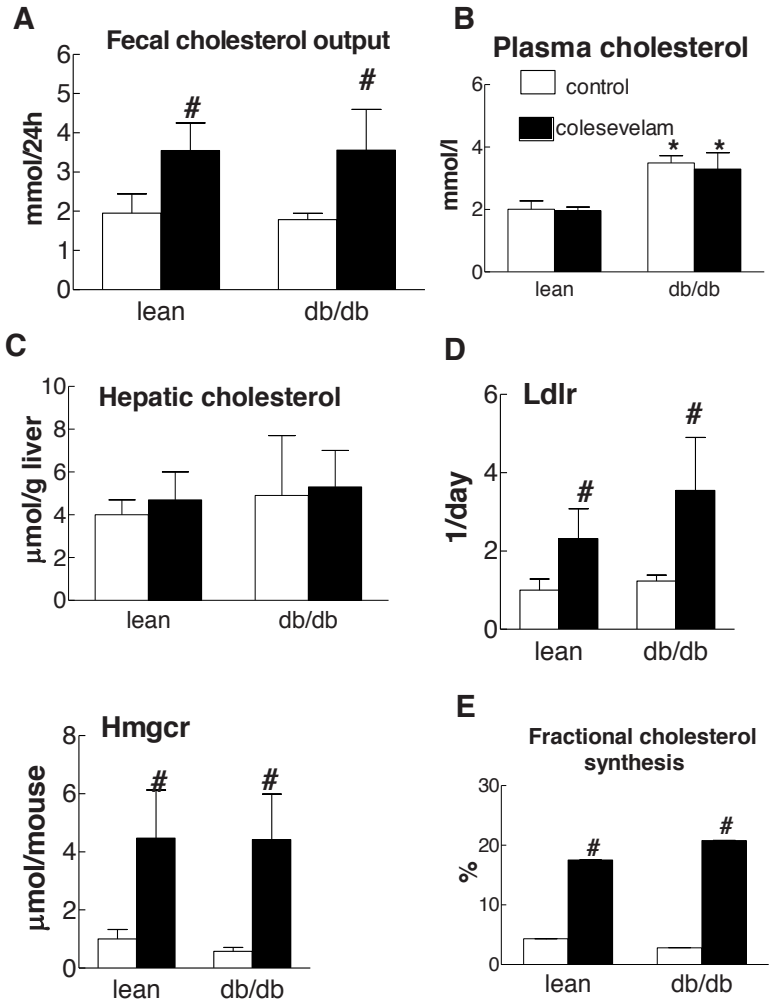


Figure 4. A massive increase in *de novo* cholesterol synthesis completely compensates for the loss of cholesterol via increased conversion into bile acids and increased fecal loss after colesevelam-treatment. Fecal cholesterol output (A) was increased in lean and *db/db* mice upon colesevelam-treatment. Yet, plasma concentrations (B) and hepatic contents (C) of cholesterol remained unchanged. Increased hepatic expression levels (D) of *Ldlr* and *Hmgcr*, key genes involved in hepatic cholesterol metabolism, strongly pointed towards compensatory cholesterol synthesis. To assess this, the fraction of newly synthesized cholesterol was calculated by means of [1-^{13}C]-acetate incorporation using Mass Isotopomer Distribution Analyses (MIDA). The fraction of newly synthesized cholesterol was indeed massively increased upon colesevelam-treatment in lean and *db/db* mice (E). Values are presented as mean ± SD. Gene expression levels are presented as relative expression normalized to *36B4*. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.

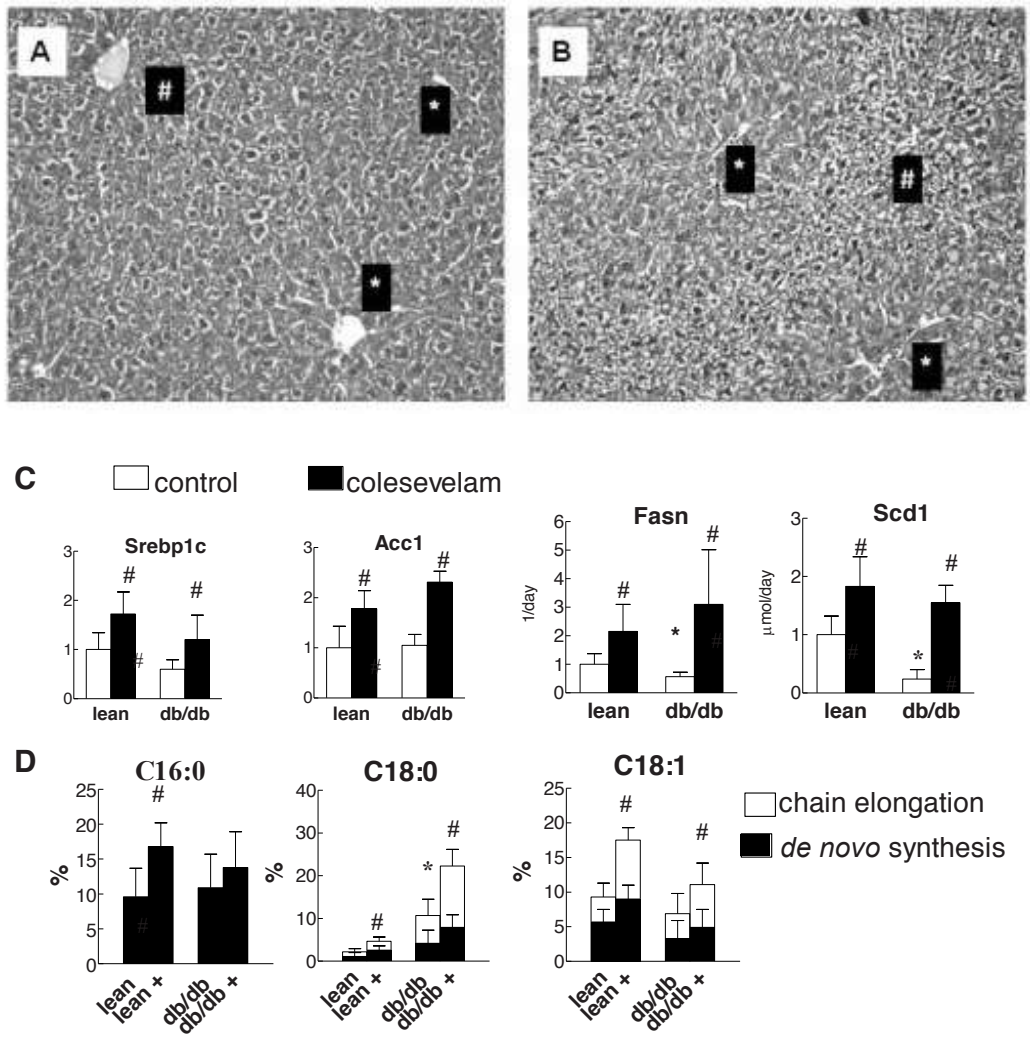
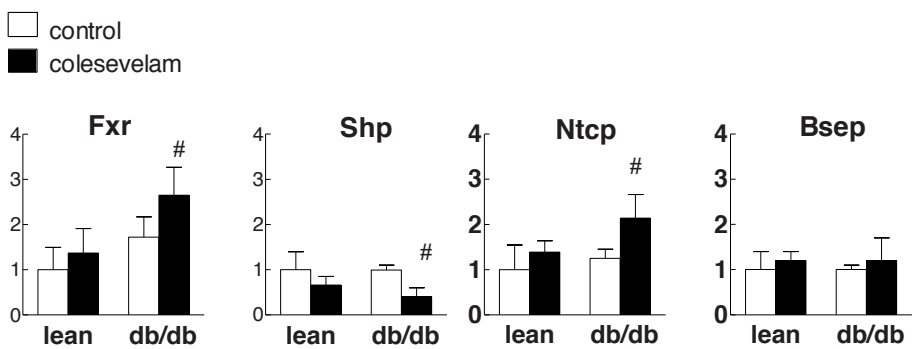


Figure 5. Increased fatty acid synthesis and chain elongation underlie the increased hepatic triglyceride content after colesevelam-treatment. Colesevelam-treatment lead to increased hepatic fat contents (Table 1) which was mainly periportally defined (A) vs (B), lean control vs lean colesevelam respectively. Increased expression of the key lipogenic genes Srebp1c, Acc1 (Acaca), Fasn and Scd1 (C) were highly suggestive of enhanced lipogenesis in colesevelam-treated mice. The fraction of newly synthesized C16:0 (D) was significantly increased in colesevelam-treated lean mice only. The fractions of newly synthesized C18:0 and C18:1 were significantly increased in lean and *db/db* mice. Additionally, chain elongation (defined as C16:0 + C:2) and desaturation were increased in Colesevelam-treated lean and *db/db* mice. Values are presented as mean ± SD. Gene expression levels are presented as relative expression normalized to *36B4*. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively. In panel D-F, * and # indicate significant differences in total (*de novo* + chain elongated) fraction of newly synthesized fatty acids. +; colesevelam-treated.

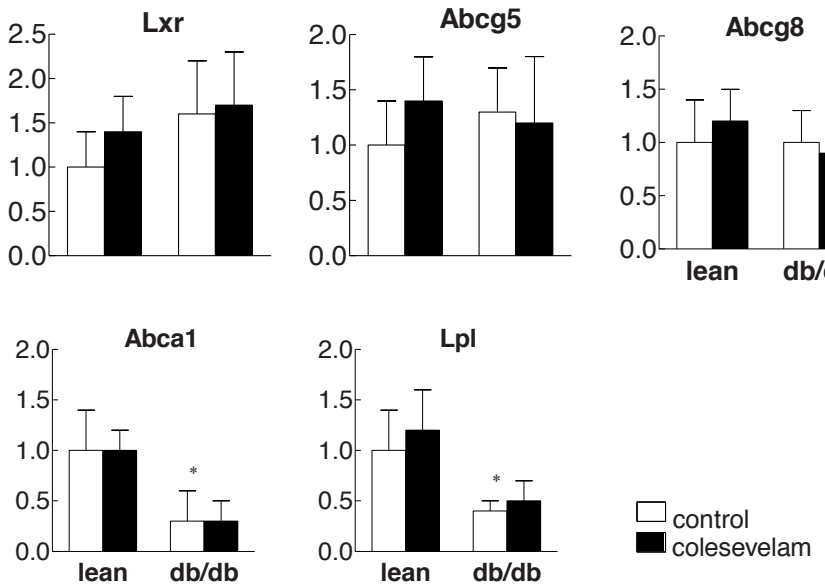
acid species was increased. Additionally, we calculated the contribution of *de novo* synthesis and chain elongation to the total fractional C18:0 and C18:1 synthesis [28]. The increased total fraction of newly synthesized fatty acids was mainly attributable to increased chain elongation in colesevelam-treated lean and *db/db* mice (Figure 5D).



Supplental Figure 2. Hepatic expression of *Fxr* and *Fxr*-target genes. Hepatic expression of Fxr (Nr1h4) and of the well-defined Fxr-target genes Shp (Nrob2), Ntcp (Slc10a1), and Bsep (Abcb11) were unaffected in colesevelam-treated lean mice compared to controls. In db/db mice, however, hepatic expression of Shp and Ntcp were reduced and increased, respectively, which is indicative of reduced FXR-signaling. Expression of Bsep was unchanged on colesevelam HCL-treated *db/db* mice compared to controls. Gene expression levels are represented as relative expression normalized to 18S. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.

Lipogenic gene expression upon sequestrant-treatment is Fxr and Lxrα dependent

Bile acid-mediated changes in expression of one of the major regulators of lipogenesis, *Srebp1c*, have been reported to be regulated by both FXR- and LXRα-regulated pathways[17]. Surprisingly, expression levels of well-defined FXR- and LXRα-target genes were differentially or not at all affected in colesevelam treated lean and *db/db* mice (Supplemental Figure 2 and 3, resp.). To further address the role of hepatic FXR and LXRα in the lipogenic response to bile acid sequestration, *Fxr*$^{-/-}$ and *Lxrα*$^{-/-}$ mice and wild-type littermates were treated with colesevelam for two weeks. The key lipogenic genes Srebp1c, Acc1, Fasn and Scd1 were significantly increased in livers of sequestrant-treated wild-type mice compared to



Supplemental Figure 3. Colesevelam-treatment does not affect hepatic expression of *Lxr (Nr1h3)* and *Lxr*-target genes. Gene expression levels are presented as relative expression normalized to *18S*. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively

untreated controls (Figure 6). Lipogenic genes, however, were barely affected in sequestrant-treated *Fxr*$^{-/-}$ and *Lxrα*$^{-/-}$ mice. These results support earlier observations on the regulatory roles of these nuclear receptors in the response to bile salt-mediated changes in lipid metabolism [17].

Discussion

This work provides novel insights in the interrelationship between bile acid and lipid metabolism in lean and diabetic *db/db* mice treated with the bile acid sequestrant colesevelam. To the best of our knowledge, this is the first report which quantitatively shows that, despite massively induced fecal bile acid loss upon sequestrant-treatment, bile acid pool sizes and biliary bile acid secretion rates remain unaffected. Additionally, we show that bile acid sequestration induces hepatic fatty acid synthesis and elongation. An altered hepatic bile acid gradient due to decreased reabsorption but increased *de novo* synthesis of bile acids likely affects specific aspects of hepatic bile acid signaling. The lipogenic response

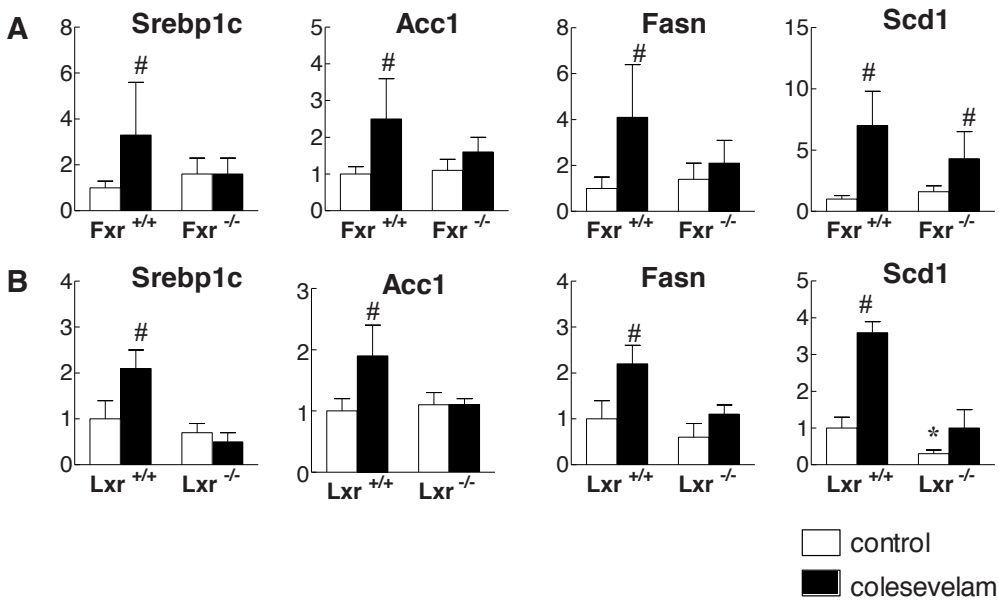


Figure 6. The lipogenic response to colesevelam treatment is dependent on FXR and LXRα signaling pathways. *Fxr*$^{-/-}$ and *Lxrα*$^{-/-}$ mice and their wild-type littermates were treated with colesevelam for two weeks. Wild-type mice had increased hepatic expression of the lipogenic genes Srebp1c, Acc1, Fasn and Scd1 upon colesevelam-treatment. On the contrary, these effects were abolished in livers of *Fxr*$^{-/-}$ and *Lxrα*$^{-/-}$ mice. Values are presented as mean ± SD. Gene expression levels are presented as relative expression normalized to *36B4*. N=4 animals per group. Significant differences within or between groups are indicated # and *, respectively.

appears to be dependent on FXR- and LXRα-signaling as was evident from studies in the respective knockout mice.

Knowledge of possible disturbances in bile acid metabolism in type 2 diabetic humans and animal models is very limited [3]. For the first time, to our knowledge, we report data on kinetic alterations of bile acid metabolism in diabetic *db/db* mice and show that *db/db* mice have an increased pool size and synthesis rate of bile acids compared to lean controls. As suggested for *db/db* mice [15] and liver-specific insulin receptor knockout (LIRKO) mice [30], disturbed hepatic insulin signaling may directly contribute to changes in bile acid synthesis. Indeed, insulin was shown to reduce plasma bile acids in type 1 diabetic rats [31] possibly via FOXO1-mediated regulation of *Cyp7a1* [32]. Further studies beyond the scope of this work, are needed to further unravel underlying mechanisms of disturbed bile acid metabolism in type 2 diabetes.

Db/db mice responded favorably to sequestrant-treatment: blood glucose levels stabilized while NEFA and VLDL-TG levels decreased. These parameters were unchanged in lean mice. Importantly, the pool size of the primary bile acid species cholate as well as the total pool size of bile acids remained unchanged in sequestrant-treated lean and *db/db* mice. Remarkably, only the synthesis of cholate was massively increased: synthesis of chenodeoxycholate-derived bile acids was not affected at all. In humans, an increased cholate-to-chenodeoxycholate ratio would result in a more hydrophilic bile acid pool which has been associated with decreased susceptibility for gallstone disease [33]. Colesevelam-treatment might therefore be beneficial for prevention of gallstone formation in type 2 diabetic humans who have an increased prevalence of gallstones[34]. Bile acid reabsorption was reduced by 30% in both models. Although bile acid reabsorption was not fully impaired, *Fgf15* expression levels were not detectable in ilea of sequestrant-treated wild-type mice. Possibly, bile acid sequestration decreases the cellular content of bile acids below a certain threshold value necessary to activate FXR in enterocytes as observed in an *in vivo* study in rabbits[35].

Interestingly, hepatic triglyceride contents of colesevelam-treated lean and *db/db* mice were enhanced which appeared to be mediated by an increased *de novo* synthesis of hepatic fatty acids and chain elongation. In contrast to our data, other studies addressing the effects of bile acid sequestration on lipid metabolism showed that bile acid sequestration prevented triglyceride accumulation in the liver [36, 37]. It should be realized that those studies were performed in high fat diet-fed mice in which the beneficial effects of bile acid sequestration are likely partly attributable to sequestrant-induced malabsorption of lipids. In addition, strain-specific responses to sequestrant-treatment cannot be ruled out.

At a molecular level, the interrelationship between bile acid and lipid metabolism is generally accepted to be mediated by FXR. Nevertheless, data to explain the exact mechanisms of this relationship are still very inconsistent. Pharmacological activation of FXR has been shown to reduce FFA levels in insulin-resistant rodents[15, 38]. Absence of FXR signaling in *Fxr*$^{-/-}$ mice leads to

increased VLDL-TG levels in plasma of these mice [39] suggestive of a role for Fxr in control of VLDL assembly.

Colesevelam-treatment induced hepatic expression levels of the lipogenic gene *Srebp1c* in lean and *db/db* mice. Hepatic expression levels of the lipogenic gene *Srebp1c* were reduced in *Fxr*$^{-/-}$ mice compared to controls [39,40]. Conversely, FXR activation was also shown to repress the expression of *Srebp1c* in a pathway involving SHP [17, 40]. Expression levels of the FXR-target gene *Shp* were unaffected and decreased in colesevelam-treated lean and *db/db* mice, respectively. These results are suggestive of SHP-independent regulation of *Srebp1c* upon sequestrant-treatment. Supportive of SHP-independent regulation of lipogenic gene expression by FXR was the observation that FXR regulates the transcription of the lipogenic gene *Fasn* through direct binding to the *Fasn* promoter [41]. Since expression levels of well-known FXR-target genes were differentially affected in lean and *db/db* mice, we studied the role of FXR in the lipogenic response of sequestrant-treatment in *Fxr*$^{-/-}$ mice and found that in contrast to wild-type mice littermates, lipogenic gene expression levels were barely affected. *Srebp1c*, is strongly regulated by the oxysterol receptor LXRα [42]. Increased synthesis of cholesterol, as occurs in sequestrant-treated mice, could possibly lead to increased hepatic levels of oxysterols and, hence, activation of LXRα. Expression levels of established LXRα-target genes, however, were unaffected in sequestrant-treated lean and *db/db* mice, suggestive of unchanged LXRα signaling. Yet, investigation in sequestrant-treated *Lxrα*$^{-/-}$ mice revealed that lipogenic gene expression was not increased in these mice compared to untreated wild-type littermates. Our results from colesevelam treated *Fxr*$^{-/-}$ and *Lxrα*$^{-/-}$ mice confirm earlier findings that FXR and LXRα are both involved in regulation of bile acid-mediated changes in lipogenic pathways [17]. The exact molecular mechanisms via which these nuclear receptors signal regulate the lipogenic response to bile acid sequestration exceed the scope of this report.

At a physiological level, bile acid-mediated signaling pathways are dependent on the concentration of bile acids in the liver acinus. We speculate that the concept of metabolic zonation [43] might add to the understanding of the observed hepatic effects upon bile acid sequestration. Hepatocytes localized around the portal vein

display different metabolic activities than those lining the central vein, *e.g.*, bile acid and fat synthesis are pericentrally localized processes whereas cholesterol synthesis is mainly carried out by portal hepatocytes [43]. As we show in the current report, the amount of bile acid molecules reabsorbed in ilea of colesevelam-treated mice was decreased by ~30% with a subsequent reduction in plasma bile acid levels and, hence, reduced bile acid signaling in periportal hepatocytes. Newly synthesized bile acids, which accommodate a much larger fraction of the bile acid pool of colesevelam-treated mice compared to controls, are primarily secreted by pericentrally localized cells and possibly exert differential signaling functions. Selective periportal fat accumulation and differentially affected expression levels of hepatic Fxr-target genes support this hypothesis. Additionally, it was shown that *Cyp7a1*, which is exclusively expressed in pericentral hepatocytes [44], translocates to a larger area of the liver lobulus with more involvement of periportally localized cells in sequestrant-treated rats [45]. It should be stressed that this hypothesis requires dedicated investigation.

In conclusion, we show that colesevelam-treatment increases lipogenesis and chain elongation in mice which, at least at the level of gene expression, is dependent on FXR and LXRα. A shift from reabsorption to *de novo* synthesis as the source of biliary bile acids affects the sinusoidal gradient of bile acids [46]. This shift modifies the regulation of genes and proteins involved in bile acid synthesis and bile acid-mediated regulation of metabolism and possibly underlies the phenotypical response to colesevelam-treatment in mice.

ACKNOWLEDGEMENTS

We are indebted to Rick Havinga for excellent contributions to the mouse studies performed. Additionally, we are grateful to Theo Boer and Elles Jonkers for their excellent technical assistance with GC/MS analyses.

RERENCES

1 Chiang, J. Y., Bile acids: regulation of synthesis, J Lipid Res, 2009, 50: 1955-1966.

2 Lefebvre, P., Cariou, B., Lien, F., Kuipers, F. and Staels, B., Role of bile acids and bile acid receptors in metabolic regulation, Physiol Rev, 2009, 89: 147-191.
3 Staels, B. and Kuipers, F., Bile acid sequestrants and the treatment of type 2 diabetes mellitus, Drugs, 2007, 67: 1383-1392.
4 Insull, W., Jr., Clinical utility of bile acid sequestrants in the treatment of dyslipidemia: a scientific review, South Med J, 2006, 99: 257-273.
5 Donovan, J. M., Von Bergmann, K., Setchell, K. D., Isaacsohn, J., Pappu, A. S., Illingworth, D. R., Olson, T. and Burke, S. K., Effects of colesevelam HC1 on sterol and bile acid excretion in patients with type IIa hypercholesterolemia, Dig Dis Sci, 2005, 50: 1232-1238.
6 Bays, H. E., Goldberg, R. B., Truitt, K. E. and Jones, M. R., Colesevelam hydrochloride therapy in patients with type 2 diabetes mellitus treated with metformin: glucose and lipid effects, Arch Intern Med, 2008, 168: 1975-1983.
7 Fonseca, V. A., Rosenstock, J., Wang, A. C., Truitt, K. E. and Jones, M. R., Colesevelam HCl improves glycemic control and reduces LDL cholesterol in patients with inadequately controlled type 2 diabetes on sulfonylurea-based therapy, Diabetes Care, 2008, 31: 1479-1484.
8 Zieve, F. J., Kalin, M. F., Schwartz, S. L., Jones, M. R. and Bailey, W. L., Results of the glucose-lowering effect of WelChol study (GLOWS): a randomized, double-blind, placebo-controlled pilot study evaluating the effect of colesevelam hydrochloride on glycemic control in subjects with type 2 diabetes, Clin Ther, 2007, 29: 74-83.
9 Beil, U., Crouse, J. R., Einarsson, K. and Grundy, S. M., Effects of interruption of the enterohepatic circulation of bile acids on the transport of very low density-lipoprotein triglycerides, Metabolism, 1982, 31: 438-444.
10 Crouse, J. R., 3rd, Hypertriglyceridemia: a contraindication to the use of bile acid binding resins, Am J Med, 1987, 83: 243-248.
11 Angelin, B., Einarsson, K., Hellstrom, K. and Leijd, B., Effects of cholestyramine and chenodeoxycholic acid on the metabolism of endogenous triglyceride in hyperlipoproteinemia, J Lipid Res, 1978, 19: 1017-1024.
12 Carulli, N., Ponz de Leon, M., Podda, M., Zuin, M., Strata, A., Frigerio, G. and Digrisolo, A., Chenodeoxycholic acid and ursodeoxycholic acid effects in endogenous hypertriglyceridemias. A controlled double-blind trial, J Clin Pharmacol, 1981, 21: 436-442.
13 Kuipers, F., Stroeve, J. H., Caron, S. and Staels, B., Bile acids, farnesoid X receptor, atherosclerosis and metabolic control, Curr Opin Lipidol, 2007, 18: 289-297.
14 Cariou, B., van Harmelen, K., Duran-Sandoval, D., van Dijk, T. H., Grefhorst, A., Abdelkarim, M., Caron, S., Torpier, G., Fruchart, J. C., Gonzalez, F. J., Kuipers, F. and Staels, B., The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice, J Biol Chem, 2006, 281: 11039-11049.
15 Zhang, Y., Lee, F. Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F. J., Willson, T. M. and Edwards, P. A., Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice, Proc Natl Acad Sci U S A, 2006, 103: 1006-1011.
16 Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G. and Gonzalez, F. J., Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis, Cell, 2000, 102: 731-744.
17 Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., Moore, D. D. and Auwerx, J., Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c, J Clin Invest, 2004, 113: 1408-1418.
18 Davidson, M. H., Dillon, M. A., Gordon, B., Jones, P., Samuels, J., Weiss, S., Isaacsohn, J., Toth, P. and Burke, S. K., Colesevelam hydrochloride (cholestagel): a new, potent bile acid sequestrant associated with a low incidence of gastrointestinal side effects, Arch Intern Med, 1999, 159: 1893-1900.
19 Kok, T., Hulzebos, C. V., Wolters, H., Havinga, R., Agellon, L. B., Stellaard, F., Shan, B., Schwarz, M. and Kuipers, F., Enterohepatic circulation of bile acids in farnesoid X

receptor-deficient mice: efficient intestinal bile acid absorption in the absence of ileal bile acid-binding protein, J Biol Chem, 2003, 278: 41930-41937.
20 Plosch, T., van der Veen, J. N., Havinga, R., Huijkman, N. C., Bloks, V. W. and Kuipers, F., Abcg5/Abcg8-independent pathways contribute to hepatobiliary cholesterol secretion in mice, Am J Physiol Gastrointest Liver Physiol, 2006, 291: G414-423.
21 Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 1959, 37: 911-917.
22 Murphy, G. M., Billing, B. H. and Baron, D. N., A fluorimetric and enzymatic method for the estimation of serum total bile acids, J Clin Pathol, 1970, 23: 594-598.
23 Hulzebos, C. V., Renfurm, L., Bandsma, R. H., Verkade, H. J., Boer, T., Boverhof, R., Tanaka, H., Mierau, I., Sauer, P. J., Kuipers, F. and Stellaard, F., Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans, J Lipid Res, 2001, 42: 1923-1929.
24 Tancevski, I., Wehinger, A., Demetz, E., Eller, P., Duwensee, K., Huber, J., Hochegger, K., Schgoer, W., Fievet, C., Stellaard, F., Rudling, M., Patsch, J. R. and Ritsch, A., Reduced plasma high-density lipoprotein cholesterol in hyperthyroid mice coincides with decreased hepatic adenosine 5'-triphosphate-binding cassette transporter 1 expression, Endocrinology, 2008, 149: 3708-3712.
25 Jung, H. R., Turner, S. M., Neese, R. A., Young, S. G. and Hellerstein, M. K., Metabolic adaptations to dietary fat malabsorption in chylomicron-deficient mice, Biochem J, 1999, 343 Pt 2: 473-478.
26 Neese, R. A., Faix, D., Kletke, C., Wu, K., Wang, A. C., Shackleton, C. H. and Hellerstein, M. K., Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA, Am J Physiol, 1993, 264: E136-147.
27 Wiegman, C. H., Bandsma, R. H., Ouwens, M., van der Sluijs, F. H., Havinga, R., Boer, T., Reijngoud, D. J., Romijn, J. A. and Kuipers, F., Hepatic VLDL production in ob/ob mice is not stimulated by massive de novo lipogenesis but is less sensitive to the suppressive effects of insulin, Diabetes, 2003, 52: 1081-1089.
28 Oosterveer, M. H., van Dijk, T. H., Tietge, U. J., Boer, T., Havinga, R., Stellaard, F., Groen, A. K., Kuipers, F. and Reijngoud, D. J., High fat feeding induces hepatic fatty acid elongation in mice, PLoS One, 2009, 4: e6066.
29 Plosch, T., Kok, T., Bloks, V. W., Smit, M. J., Havinga, R., Chimini, G., Groen, A. K. and Kuipers, F., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1, J Biol Chem, 2002, 277: 33870-33877.
30 Biddinger, S. B., Haas, J. T., Yu, B. B., Bezy, O., Jing, E., Zhang, W., Unterman, T. G., Carey, M. C. and Kahn, C. R., Hepatic insulin resistance directly promotes formation of cholesterol gallstones, Nat Med, 2008, 14: 778-782.
31 van Waarde, W. M., Verkade, H. J., Wolters, H., Havinga, R., Baller, J., Bloks, V., Muller, M., Sauer, P. J. and Kuipers, F., Differential effects of streptozotocin-induced diabetes on expression of hepatic ABC-transporters in rats, Gastroenterology, 2002, 122: 1842-1852.
32 Li, T., Ma, H. and Chiang, J. Y., TGFbeta1, TNFalpha, and insulin signaling crosstalk in regulation of the rat cholesterol 7alpha-hydroxylase gene expression, J Lipid Res, 2008, 49: 1981-1989.
33 Hay, D. W. and Carey, M. C., Pathophysiology and pathogenesis of cholesterol gallstone formation, Semin Liver Dis, 1990, 10: 159-170.
34 Ruhl, C. E. and Everhart, J. E., Association of diabetes, serum insulin, and C-peptide with gallbladder disease, Hepatology, 2000, 31: 299-303.
35 Xu, G., Pan, L. X., Li, H., Forman, B. M., Erickson, S. K., Shefer, S., Bollineni, J., Batta, A. K., Christie, J., Wang, T. H., Michel, J., Yang, S., Tsai, R., Lai, L., Shimada, K., Tint, G. S. and Salen, G., Regulation of the farnesoid X receptor (FXR) by bile acid flux in rabbits, J Biol Chem, 2002, 277: 50491-50496.
36 Kobayashi, M., Ikegami, H., Fujisawa, T., Nojima, K., Kawabata, Y., Noso, S., Babaya, N., Itoi-Babaya, M., Yamaji, K., Hiromine, Y., Shibata, M. and Ogihara, T.,

Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin, Diabetes, 2007, 56: 239-247.
37 Sakamoto, S., Takeshita, S., Sassa, S., Suzuki, S., Ishikawa, Y. and Kudo, H., Effects of colestimide and/or Bofu-tsusho-san on plasma and liver lipids in mice fed a high-fat diet, In Vivo, 2005, 19: 1029-1033.
38 Bilz, S., Samuel, V., Morino, K., Savage, D., Choi, C. S. and Shulman, G. I., Activation of the farnesoid X receptor improves lipid metabolism in combined hyperlipidemic hamsters, Am J Physiol Endocrinol Metab, 2006, 290: E716-722.
39 Duran-Sandoval, D., Cariou, B., Percevault, F., Hennuyer, N., Grefhorst, A., van Dijk, T. H., Gonzalez, F. J., Fruchart, J. C., Kuipers, F. and Staels, B., The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition, J Biol Chem, 2005, 280: 29971-29979.
40 Zhang, Y., Castellani, L. W., Sinal, C. J., Gonzalez, F. J. and Edwards, P. A., Peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC-1alpha) regulates triglyceride metabolism by activation of the nuclear receptor FXR, Genes Dev, 2004, 18: 157-169.
41 Matsukuma, K. E., Bennett, M. K., Huang, J., Wang, L., Gil, G. and Osborne, T. F., Coordinated control of bile acids and lipogenesis through FXR-dependent regulation of fatty acid synthase, J Lipid Res, 2006, 47: 2754-2761.
42 Grefhorst, A., van Dijk, T. H., Hammer, A., van der Sluijs, F. H., Havinga, R., Havekes, L. M., Romijn, J. A., Groot, P. H., Reijngoud, D. J. and Kuipers, F., Differential effects of pharmacological liver X receptor activation on hepatic and peripheral insulin sensitivity in lean and ob/ob mice, Am J Physiol Endocrinol Metab, 2005, 289: E829-838.
43 Jungermann, K. and Kietzmann, T., Zonation of parenchymal and nonparenchymal metabolism in liver, Annu Rev Nutr, 1996, 16: 179-203.
44 Wang, J., Olin, M., Rozell, B., Bjorkhem, I., Einarsson, C., Eggertsen, G. and Gafvels, M., Differential hepatocellular zonation pattern of cholesterol 7alpha-hydroxylase (Cyp7a1) and sterol 12alpha-hydroxylase (Cyp8b1) in the mouse, Histochem Cell Biol, 2007, 127: 253-261.
45 Twisk, J., Hoekman, M. F., Mager, W. H., Moorman, A. F., de Boer, P. A., Scheja, L., Princen, H. M. and Gebhardt, R., Heterogeneous expression of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus, J Clin Invest, 1995, 95: 1235-1243.
46 Groothuis, G. M. and Meijer, D. K., Hepatocyte heterogeneity in bile formation and hepatobiliary transport of drugs, Enzyme, 1992, 46: 94-138.

Supplemental Table 1. Fecal and biliary bile acid composition as percentage of total bile acids. Secondary bile acids are marked *. Values are presented as mean ± SD. N=6 animals per group. +; colesevelam-treated

	α-M	DC *	CA	allo-CA	CDC	HDC *	UDC *	β-M	ω-M *
Fecal bile salts									
Lean control	2.4 ± 0.3	10.0 ±1.4	62.5 ± 4.4	4.9 ± 1.0	2.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.4	6.1 ± 1.8	15.1±3.6
Lean +	4.6 ± 0.5	74.1 ± 6.1	11.1 ± 5.0	1.7 ± 0.3	1.6 ± 0.4	0.5 ± 0.1	0.7 ± 0.1	4.0 ± 0.8	3.4 ±1.4
db/db control	7.1 ± 2.8	28.7 ± 8.0	37.1 ± 9.7	2.0 ± 0.3	1.9 ± 0.4	0.9 ± 0.1	1.0 ± 0.3	7.3 ± 0.5	13.6 ±5.3
db/db +	5.2 ± 0.9	60.7 ± 9.4	23.9 ± 9.7	1.1 ± 0.4	5.5 ± 2.7	0.2 ± 0.1	0.4 ± 0.2	2.1 ± 0.5	1.0 ± 0.7
Biliary bile salts									
Lean control	2.8 ± 0.2	1.8 ± 0.2	48.0 ± 3.9	8.8 ±1.4	1.8 ± 0.2	1.3 ± 0.2	2.9 ± 0.6	18.9 ± 2.8	13.8 ± 3.8
Lean +	3.3 ± 0.3	3.5 ± 0.6	64.5 ± 3.3	5.2 ± 0.3	3.3 ± 0.2	0.7 ± 0.8	1.9 ± 0.6	13.1± 2.4	4.4 ± 1.1
db/db control	2.0 ± 0.5	1.2 ± 0.2	67.6 ± 8.4	3.2 ± 0.8	1.5 ± 0.1	0.9 ± 0.2	1.3 ± 0.3	13.6 ± 5.8	8.7 ± 2.8
db/db +	3.0 ± 0.6	1.7 ± 0.3	78.4 ± 3.7	2.9 ± 0.5	3.8 ± 0.8	1.0 ± 0.5	1.5 ± 0.2	5.7 ± 1.2	2.2 ± 0.7

CHAPTER 3

Bile acid sequestration reduces plasma glucose levels in *db/db* mice by increasing its metabolic clearance rate

M. Meissner, H. Herrema, T. H. v. Dijk, A. Gerding, R. Havinga, T. Boer, M. Müller, D-J Reijngoud, A. K. Groen, F. Kuipers

Submitted

ABSTRACT

Aims/hypothesis Bile acid sequestrants (BAS) reduce plasma glucose levels in type II diabetics and in murine models of diabetes but the mechanism herein is unknown. We hypothesized that sequestrant-induced changes in hepatic glucose metabolism would underlie reduced plasma glucose levels. Therefore, *in vivo* glucose metabolism was assessed in *db/db* mice on and off BAS using tracer methodology.

Methods Lean and diabetic *db/db* mice were treated with 2% (wt/wt in diet) Colesevelam HCl (BAS) for 2 weeks. Parameters of *in vivo* glucose metabolism were assessed by infusing [U-^{13}C]-glucose, [2-^{13}C]-glycerol, [1-^{2}H]-galactose and paracetamol for 6 hours, followed by mass isotopomer distribution analysis, and related to metabolic parameters as well as gene expression patterns.

Results Compared to lean mice, *db/db* mice displayed an almost 3-fold lower metabolic clearance rate of glucose ($p=0.0001$), a ~3-fold increased glucokinase flux ($p=0.001$) and a ~2-fold increased total hepatic glucose production rate ($p<0.001$). BAS treatment increased glucose metabolic clearance rate by ~37% but had no effects on glucokinase flux nor total hepatic or endogenous glucose production. Strikingly, BAS-treated *db/db* mice displayed reduced long-chain acylcarnitine content in skeletal muscle ($p=0.032$) but not in liver ($p=0.189$). Unexpectedly, BAS treatment increased hepatic FGF21 mRNA expression 2-fold in lean mice ($p=0.030$) and 3-fold in *db/db* mice ($p=0.002$).

Conclusions/interpretation BAS induced plasma glucose lowering in *db/db* mice by increasing metabolic clearance rate of glucose in peripheral tissues, which coincided with decreased skeletal muscle long-chain acylcarnitine content. BAS-mediated induction of hepatic FGF21 might play a modulating role herein.

INTRODUCTION

Type 2 diabetes is a major health problem worldwide [1]. The predominant features of type 2 diabetes entail increased fasting blood glucose levels, increased plasma triglycerides and LDL-cholesterol levels, as well as disturbed peripheral glucose utilization [2-4]. The use of bile acid sequestrants (BAS) for lowering of LDL-cholesterol levels is well-established [5-7]. More recently, Colesevelam HCl, a BAS, has been indicated by the FDA to improve glycemic control in patients with type 2 diabetes [8-10]. So far, however, the actual changes in hepatic and/or peripheral glucose metabolism upon BAS supplementation are not understood.

Bile acids are synthesized from cholesterol in the liver. Upon secretion into bile, bile acids function to emulsify fats in the small intestine. Most of the secreted biliary bile acids are reabsorbed in the ileum (enterohepatic circulation). Sequestrants interfere with the enterohepatic circulation of bile acids by binding them in the intestine, thereby inhibiting their reabsorption and promoting their fecal loss. As a consequence, the liver increases bile acid synthesis and subsequently cholesterol uptake from the circulation thereby reducing LDL-cholesterol levels [11, 12].

To date, there is a lack of understanding how BAS reduce plasma glucose levels. However, a large body of research suggests that bile acids modulate hepatic glucose metabolism via signaling pathways mediated by the nuclear receptor Fxr in diabetes [13-19]. Fxr is expressed in the liver, intestine, adrenal gland and kidney [20], and it acts to inhibit *de novo* bile acid synthesis when activated by bile acids in the liver [21]. Paradoxically, both agents that inhibit *de novo* bile acid synthesis, such as bile acids themselves and synthetic Fxr ligands [14, 22], as well as agents that increase *de novo* bile acid synthesis such as BAS, were shown to reduce plasma glucose levels in diabetic mice [23, 24]. Thus, the cause of bile acid-mediated changes in blood glucose levels remains elusive.

We therefore questioned whether BAS-induced changes in hepatic carbohydrate fluxes are responsible for the observed reduction in plasma glucose levels in *db/db* mice. To test this, we treated healthy lean mice and obese, diabetic *db/db* mice with Colesevelam HCl. Applying an *in vivo* infusion protocol of stable

isotopes followed by mass isotopomer distribution analysis (MIDA), we first characterized specific disruptions of whole body glucose turnover and hepatic glucose metabolism in *db/db* mice. We then tested the hypothesis that BAS restores disrupted hepatic glucose fluxes thereby mediating the previously observed reduction in blood glucose levels in diabetic mice. In view of the strong interaction between glucose and fatty acid metabolism, we additionally tested the effect of BAS on levels of lipids and intermediates of fatty acid metabolism in liver and muscle.

METHODS and MATERIALS

Animals and diets

Ten week old male lean C57BL/6 and obese, diabetic *db/db* mice on a C57BL/6 background (B6.Cg-*m* +/+ *Lepr*^{db}/J) were purchased from Charles River Laboratories (L'Arbresle, France and Brussels, Belgium, respectively). Mice were housed in a temperature controlled (21°C) room with a dark-light cycle of 12 h each. For all animal experiments the principles of laboratory animal care (NIH publication no. 85–23, revised 1985) were followed. The Ethics Committee for Animal Experiments of the University Groningen, the Netherlands, approved experimental procedures.

One week after arrival at the animal facility, 8 *db/db* (db) and 6 lean (L) mice were put on a diet containing standard laboratory chow (RMH-B; Arie Blok, Woerden, The Netherlands) supplemented with 2% (wt/wt) Colesevelam HCl (Daiichi Sankyo, Inc., Parsippany, NJ, USA) for 2 weeks. Another 8 *db/db* and 6 lean mice remained on standard laboratory chow. Body weights and food intake were recorded every other day. One week into the diets, mice were fitted with a permanent catheter in the right atrium via the jugular vein, as described previously [25]. Mice were allowed to recover from surgery for 6 days.

Materials

The following isotopes were used: [2-^{13}]-C glycerol (99% ^{13}C atom percent excess), [1-^{2}H]-galactose (98% ^{2}H atom percent excess) (Isotec, Miamisburg, Ohio, USA),

[U-^{13}C]-glucose (99% ^{13}C atom percent) (Cambridge Isotope Laboratories, Andover, Mass., USA). All reagents and chemicals used were reagent pro analysis grade. Blood spots and urine were collected on Schleicher and Schuell No. 2992 filter paper (Schleicher and Schuells, 's Hertogenbosch, The Netherlands). Infusates were freshly prepared and sterilized at the day before the experiment.

Animal Experiments

The infusion experiment was performed in conscious mice, as described previously [25]. Mice were fasted for 4 hours (03:00-08:00 am) and then housed in metabolic cages to allow frequent collection of urine and blood spots on filter paper. Mice were infused with a sterile solution containing [U-^{13} C]-glucose (13.9 μmol/ml), [2-^{13}C]-glycerol (160 μmol/ml), [1-^{2}H]-galactose (33 μmol/ml) and paracetamol (1.0mg/ml) at a rate of 0.6 ml/h. Before and during the experiment, small blood samples were obtained via tail bleeding to allow for the determination of plasma glucose. Blood was immediately centrifuged and stored at -20°C until analysis. Blood spots were collected on filter paper before the start of the infusion and hourly afterwards until 6 h after the start of the infusion. Blood spots were air dried and stored at room temperature until analysis. Hourly urine samples were collected on filter paper, air dried and stored at room temperature until analysis. At the end of the experiment, animals were anesthetized with isoflurane and a small blood sample was collected via orbital puncture for the determination of insulin.

Mice were allowed to recover and five days after the infusion experiment. Mice were then fasted for 7 hours (03:00-10:00h) and terminated by heart puncture under isoflurane anesthesia. A large blood sample was collected in heparin-containing tubes, immediately centrifuged and stored at -20 °C until analysis. Liver was excised, weighed, snap frozen and stored at -80 °C until further analysis. Skeletal muscle was collected, frozen in liquid N_2 and stored at -80 °C until further analysis.

Measurement and Analysis of Mass Isotopologue Distribution Analysis by GC-MS

Analytical procedures for extraction of glucose in bloodspots and paracetamol-glucuronide from urine filter paper strips and derivatization of the extracted compounds and GC-MS measurements of derivatives were all performed according to Van Dijk *et al* [26, 27]. The measured fractional distribution was corrected for natural abundance of ^{13}C by multiple linear regression as described by Lee *et al*. [28] to obtain the excess mole fraction of mass isotopologues due to incorporation and dilution of infused labeled compounds, i.e., [2-^{13}C]-glycerol, [U-^{13}C]-glucose and [1-^{2}H]-galactose. This distribution was used in mass isotopologues distribution analysis (MIDA) algorithms of isotope incorporation and dilution according to Hellerstein *et al*.[29] as described by Van Dijk *et al*.[26, 27].

Determination of metabolite concentrations

Commercially available kits were used to determine plasma levels of insulin (Mercodia, Uppsala, Sweden), triglycerides, total cholesterol, free cholesterol and NEFA (Wako Chemicals, Neuss, Germany),

Plasma HOMA-index was calculated multiplying the blood glucose levels by the plasma insulin levels at 6h of MIDA infusion and dividing the product by 61.64, which was the HOMA-reference factor derived from the product of the average blood glucose level and plasma insulin level of lean control mice of this experiment. Hepatic glycogen and glucose-6-phosphate content were determined as previously described [25]. Hepatic lipids were determined in liver homogenates by commercially available kits for triglycerides and total cholesterol (Wako Chemicals, Neuss, Germany) after lipid extraction as described by Bligh and Dyer [30]. Plasma acylcarnitines were determined according to the method of Chase *et al* [31] as described by Derks *et al*.[32]. Profiles of long-chain acylcarnitines (C16:0, C16:1, C18:0, C18:1 and C18:2) in muscle and liver homogenates (15% (w/v) in PBS) were determined according to the method of Gates [33].

mRNA levels

Total RNA was isolated from liver using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described by Plösch and coworkers [34]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwerkerk ad IJssel, The Netherlands). Primer and probe sequences have been deposited at the RTprimerDB [35]. PCR results were normalized to 18S-rRNA abundance.

Statistics

All values are represented as mean ± standard deviation. Statistical significance was assessed using the Mann-Whitney-U-test (SPSS 12.0.1 for Windows). P-values were corrected for multiple comparison errors. Statistical significance was accepted for a $p<0.05$.

RESULTS

Bile acid sequestration reduced plasma glucose levels in *db/db* mice.

Previously, our laboratory has shown that Colesevelam HCl treatment lowers plasma glucose concentrations in *db/db* mice compared to untreated counterparts [24]. To confirm these findings, basal parameters related to glucose metabolism were determined in lean and *db/db* mice treated with the bile acid sequestrant (BAS) for two weeks. As expected, BAS-treatment lowered blood glucose levels of diabetic mice *(Table 1)*. No effects of BAS on body weight, liver weight or liver weight / body weight ratio were observed (Table 1). Plasma insulin concentrations were decreased (~30%) and the HOMA-index was improved in sequestrant-treated diabetic mice compared to untreated counterparts. Moreover, we observed a more than 50% reduction in plasma 3-hydroxybutyrate levels and a non-significant decrease in plasma NEFA levels (Table 1). Consistent with our previous observations [24], liver triglyceride contents were increased in sequestrant-treated lean and *db/db* mice compared to controls (Table 1).

Table 1. 7h fasted parameters (unless otherwise stated) in lean mice (L), lean mice supplemented with BAS (L BAS), *db/db* (db) and *db/db* mice supplemented with BAS (db BAS). Data are shown as means ± SD; *p<0.05 vs. same genotype, †p<0.05 vs. lean same condition

	L	L BAS	db	db BAS
Body weight (g)	23.9 ± 1.1	24.9 ± 0.9	37.9 ± 3.2†	39.7 ± 3.5†
Liver weight (g)	1.14 ± 0.08	1.19 ± 0.09	1.74 ± 0.24†	1.93 ± 0.27†
Liver weight / Body weight (%)	4.7 ± 0.2	4.7 ± 0.2	4.7 ± 0.6	4.9 ± 0.7
Plasma Parameters				
4h fasted blood glucose (mmol/L)	8.3 ± 0.6	8.8 ± 0.9	27.7 ± 4.4†	19.3 ± 4.8†*
Blood glucose at end of MIDA infusion (mmol/L)	9.2 ± 0.5	9.7 ± 1.0	32.7 ± 3.9†	23.1 ± 3.9†*
Plasma insulin at end of MIDA infusion (mU/L)	6.7 ± 1.8	7.5 ± 1.6	66.6 ± 23.3†	48.0 ± 28.4†
HOMA Index	1.0 ± 0.3	1.2 ± 0.3	34.8 ± 10.1†	16.9 ± 6.7†*
NEFA (µmol/L)	300 ± 109	393 ± 92	697 ± 149†	555 ± 149
Lactate (mmol/L)	8.4 ± 1.1	8.3 ± 1.3	6.8 ± 1.5	8.6 ± 1.8
3-Hydroxybutyrate (mmol/L)	0.3 ± 0.2	0.3 ± 0.2	1.5 ± 1.0†	0.7 ± 0.3†
Triglycerides (mmol/L)	0.7 ± 0.2	0.5 ± 0.1*	1.0 ± 0.1	0.7 ± 0.1 *
Total cholesterol (mmol/L)	2.3 ± 0.3	2.3 ± 0.2	2.8 ± 0.1 †	3.2 ± 0.4†*
Liver Parameters				
Triglycerides (µmol/g liver)	16 ± 6	31 ± 14*	44 ± 11†	59 ± 21†
Glycogen (µmol/g liver)	62 ± 45	47 ± 30	175 ± 21†	216 ± 37†
Glucose-6-Phosphate (nmol/mg liver)	0.69 ± 0.18	0.74 ± 0.18	0.40 ± 0.16†	0.42 ±0.10†

Bile acid sequestration increased metabolic clearance of glucose without affecting hepatic glucose production.

BAS promotes specific changes in hepatic cholesterol and bile acid synthesis [24]. To gain insight in hepatic glucose metabolism upon BAS-treatment, *in vivo* glucose metabolism was studied in lean and *db/db* mice after 2 weeks of BAS treatment. First, whole body and hepatic glucose metabolism in *db/db* mice was characterized. In *db/db* mice, plasma glucose concentrations were almost 3 times higher than in lean mice (9.2 ± 0.5, 32.7 ± 3.9 mmol/l, lean *vs. db/db,* Table 1). The rate of uptake of plasma glucose by peripheral tissues was only slightly but significantly higher in *db/db* mice compared to lean mice (93.1. ± 13.1 vs. 117.0 ± 14.6 $ml.kg^{-1}.min^{-1}$,

lean *vs. db/db,* Table 2). Consequently, the metabolic clearance rate of plasma glucose was lower in *db/db* mice (9.9 ± 1.3 vs. 3.6 ± 0.7 $ml.kg^{-1}.min^{-1}$, lean *vs. db/db, Table 2*). Diabetic mice had significantly higher hepatic glycogen contents compared to lean mice while, surprisingly, hepatic glucose-6-phosphate contents was decreased in the *db/db* genotype (Table 1). A strongly increased hepatic glucose cycling brought about by a massively increased glucokinase flux was observed in *db/db* compared to lean mice (47 ± 5 *vs.* 150 ± 58 $umol.kg^{-1}.min^{-1}$, lean *vs. db/db,* Table 2). However, since hepatic clearance of glucose by glucokinase, as derived by dividing blood glucose from the glucokinase flux, was not significantly different from hepatic glucose clearance in lean mice (5.1 ± 0.7 $ml.kg^{-1}.min^{-1}$ vs. 4.6 ± 0.7 $ml.kg^{-1}.min^{-1}$, lean vs. *db/db* mice), the observed increase in glucose cycling was most likely driven by the extreme hyperglycemia in *db/db* mice. In *db/db* mice, *de novo* synthesis of glucose-6-phosphate was significantly decreased when compared to lean mice (86 ± 6 $umol.kg^{-1}.min^{-1}$ *vs.* 74 ± 11 $umol.kg^{-1}.min^{-1}$, lean *vs. db/db*, $p<0.05$, Table 2). Endogenous glucose production (excluding glucose cycling) was slightly higher (87 ± 13 vs. 115 ± 14 $umol.kg^{-1}.min^{-1}$, lean *vs. db/db*, $p<0.05$, Table 2) whereas total glucose output (including glucose cycling) was massively higher in *db/db* compared to lean mice (118 ± 17 $umol/kg^{-1}.min^{-1}$ vs. 244 ± 54. $umol/kg^{-1}.min^{-1}$ lean and *db/db* mice, respectively (Table 2).

BAS treatment had differential effects on whole body glucose metabolism in lean and *db/db* mice. Irrespective of the decreased plasma glucose concentration, BAS treatment had no effect on the flux through glucokinase in livers of *db/db* mice. As a consequence, hepatic clearance of glucose by glucokinase was significantly increased in *db/db* compared to lean mice, from 5.6 ± 0.4 $ml.kg^{-1}.min^{-1}$ in lean mice to 8.1 ± 0.7 $ml.kg^{-1}.min^{-1}$ in *db/db* mice ($p<0.05$). Since the glucokinase flux remained high, glucose cycling remained higher in treated *db/db* mice when compared to treated lean mice. In treated *db/db* mice the rate of endogenous glucose production did not change and the rate of total hepatic glucose output tended to increase, albeit non-significantly (Table 2). Although BAS was not effective to reduce rates of hepatic glucose consumption or production, we did observe a decrease of *de novo* glucose-6-phosphate synthesis in treated *db/db*

Table 2. *In vivo* parameters of hepatic glucose metabolism during the last 3 hours of the infusion experiment in lean mice (L), lean mice supplemented with BAS (L BAS), *db/*db mice (db) and *db/db* mice supplemented with BAS (db BAS). The middle panel of the table shows the contributions of *de* novo glucose-6-phosphate synthesis to glucose and of glycogen to glucose to the endogenous glucose production rate. The middle panel shows the contributions of the endogenous glucose production rate and the glucose cycling rate to the total hepatic glucose production rate which equals the flux rate through glucose-6-phosphatase. The lower panel shows the flux rates through glucokinase and the rate of glucose-6-phosphate *de novo* synthesis. Each value represents the mean ± SD; *p<0.05 vs. same genotype untreated; †p<0.05 vs. L same condition

	L	LBAS	db	db BAS
Rate of glucose disposal (μmol/kg/min)	93 ± 13	107 ± 9.2	117 ± 13†	119 ± 8†
Metabolic clearance rate (ml/kg/min)	9.9 ± 1.3	10.7 ± 0.6	3.6 ± 0.7†	5.3 ± 0.3†*
	Contributions to endogenous glucose production rate (μmol/kg/min)			
De novo glucose-6-phosphate synthesis to glucose	60 ± 7	64 ± 8	66 ± 6	56 ± 8*
Glycogen to glucose	27 ± 6	37 ± 5*	49 ± 8†	61 ± 6†*
Endogenous glucose production rate	87 ± 13	101 ± 9	115 ± 14†	117 ± 6†
	Contributions to hepatic glucose production rate (μmol/kg/min)			
Endogenous glucose production rate	87 ± 13	101 ± 9	115 ± 14†	117 ± 6†
Glucose cycling rate	31 ± 4	38 ± 3	129 ± 53†	156 ± 53†
Total hepatic glucose production rate	118 ± 17	139 ± 17	244 ± 54†	273 ± 58†
	Flux rates (μmol/kg/min)			
Glucokinase	47 ± 5	56 ± 7	150 ± 58†	184 ± 54†
Glucose-6-Phosphate *de novo* synthesis	86 ± 6	87 ± 9	74 ± 11	61 ± 7†*

mice (87 ± 9 μmol/kg^{-1}.min^{-1} *vs*. 61 ± 7 μmol/kg^{-1}.min^{-1}, lean vs. *db/db* mice, $p<0.05$, Table 2). This accounted for the decreased contribution of newly synthesized glucose-6-phosphate towards plasma glucose *(*Table 2). Since the flux through glucokinase and glucose cycling remained invariantly high, these decreases of *de novo* glucose-6-phosphate synthesis and newly synthesized glucose-6-phosphate partitioning towards glucose did not translate into a decreased total glucose output in *db/db* mice. Importantly, BAS treatment significantly increased peripheral metabolic clearance of glucose in *db/db* mice (~37%*;* Table 2) without affecting whole body glucose disposal (Table 2). Improved plasma glucose concentrations in BAS-treated *db/db* mice are therefore mainly attributed to increased peripheral glucose clearance.

To assess whether changes in hepatic glucose metabolism were in parallel with changes in gene expression patterns, expression levels of genes involved in hepatic glucose metabolism in untreated lean and *db/db* mice were compared. Compared to lean mice, in *db/db* mice expression levels of glucose transporter 2, glucokinase and glucose-6-phosphate hydrolase were increased, whereas no difference was observed in the expression of the gene encoding phospho-*enol*-pyruvate carboxykinase (Figure 1A, B, C and D). Furthermore, expression of the glycolytic enzyme pyruvate kinase was not differentially expressed in lean and *db/db* mice *(Figure 1E).* Additionally, expression levels of these genes were measured in both models following BAS-treatment. BAS treatment had differential effects on expression of genes involved in hepatic glucose metabolism. In lean mice, expression of glucose transporter 2 and glucokinase was increased upon BAS treatment whereas expression levels remained high in *db/db* mice. Quite surprisingly, expression of the glycolytic enzyme pyruvate kinase was strongly increased upon treatment in *db/db* mice whereas expression levels remained unaffected in lean mice upon treatment. (Figure 1E). In addition, hepatic gene expression levels of fibroblast growth factor 21 (FGF21), which has lately gained attention for its remarkable *in vivo* actions on glucose metabolism, were measured [36]. Surprisingly, expression levels of FGF21 were 2-fold increased in treated lean and 3- fold increased in treated *db/db* mice compared to untreated counterparts (Figure 1F).

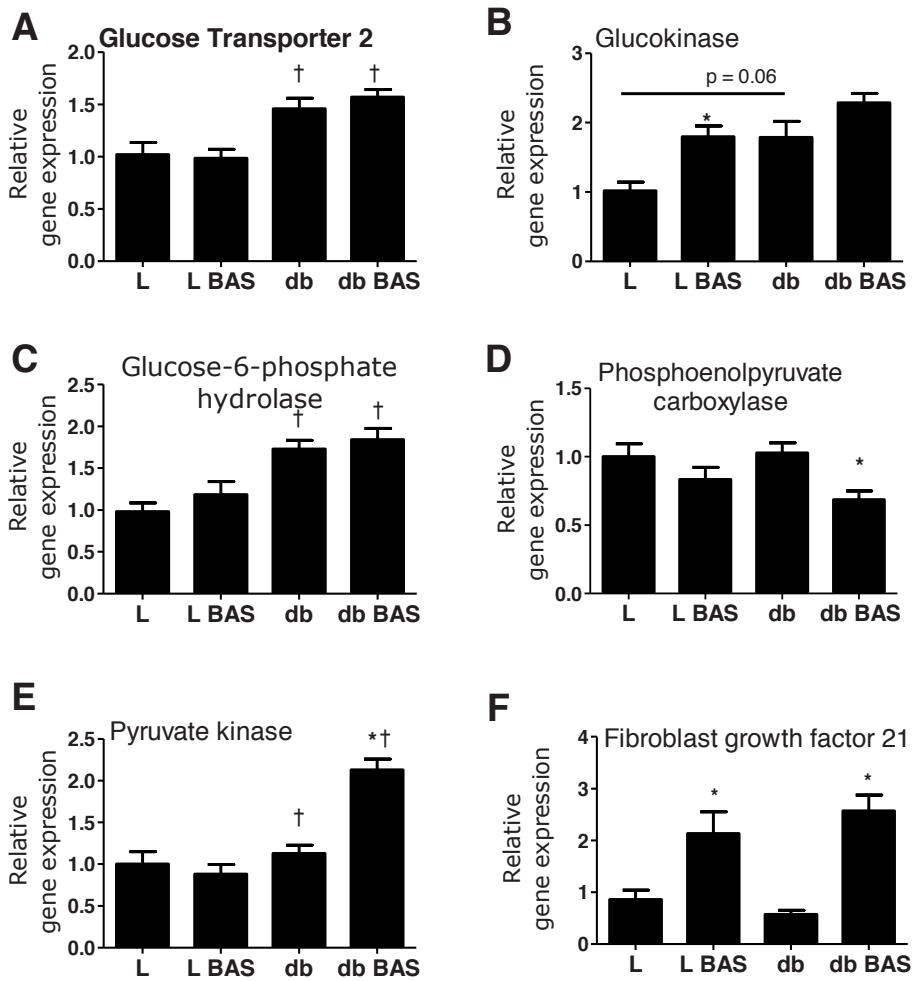


Figure 1. Hepatic mRNA expression levels of genes involved in hepatic glucose metabolism in lean mice (L, n=5), lean mice supplemented with BAS (L BAS, n=6), db/db mice (db, n=8) and db/db mice supplemented with BAS (db BAS, n=8) for glucose transporter 2 (A), glucokinase (B), glucose-6-phosphate hydrolase (C), phospho-enol pyruvate carboxykinase (D), pyruvate kinase (E) and fibroblast growth factor 21 (F). Expression of genes was normalized to 18S-rRNA. 18S-rRNA levels were similar in livers of all animals.Data are mean ± SD; *p<0.05 vs. same genotype untreated; †p<0.05 vs. L same condition

Bile acid sequestration reduced long-chain acylcarnitine content in muscle and plasma in *db/db* mice.

Skeletal muscle is the major site of both glucose and fatty acid uptake and oxidation [4]. It is known that under circumstances of high glucose concentrations

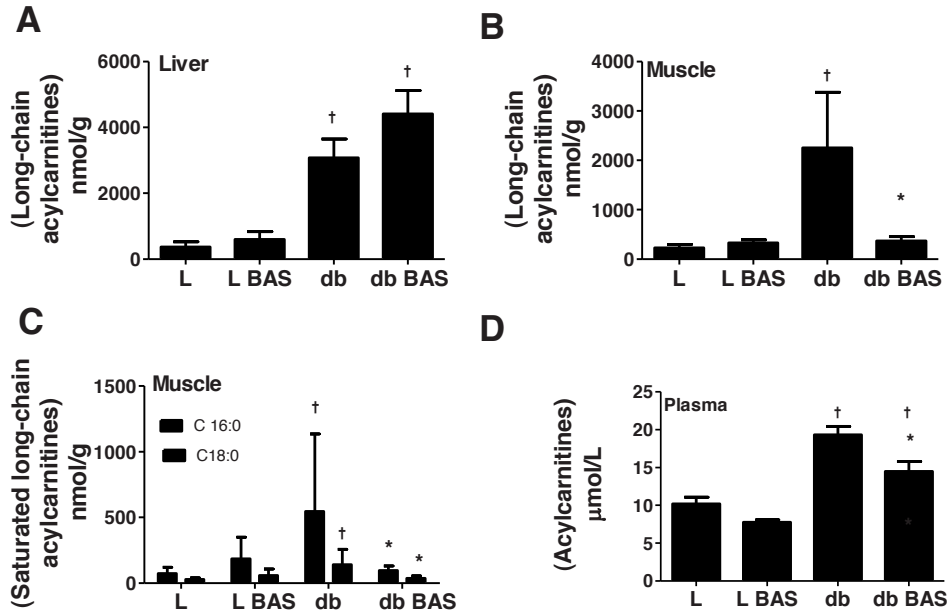


Figure.2. Effect of BAS on hepatic long-chain acylcarnitines content (Sum of C16:0, C16:1, C18:0, C18:1 and C18:2) (A), skeletal muscle long-chain acylcarnitines content (B), skeletal muscle saturated long-chain acylcarnitines content (C) and plasma acylcarnitine concentration (D) in lean mice (L, (n=5), lean mice supplemented with BAS (L BAS, n=4), db/db mice (db, n=5) and db/db mice supplemented with BAS (db BAS, n=5). Data are mean ± SD; † $p<0.05$ vs. L same condition; * $p<0.05$ vs. same genotype untreated

muscle favors glucose uptake and oxidation over fatty acid uptake and oxidation [4], while accumulating excess fatty acids, in particular saturated free fatty acid species like stearate and palmitate [37, 38]. Recently, it has been shown that excessive fatty acid oxidation in diabetic mice results in inefficient oxidation [39]. Concomitantly, high intracellular concentrations of long-chain acylcarnitines, markers of inefficient mitochondrial fatty acid oxidation, were measured. High concentrations of these intermediates can impair the switch to carbohydrate oxidation, a marker of "metabolic flexibility". To study whether BAS affected fatty acid metabolism, long-chain acylcarnitine contents in liver and skeletal muscle were measured. In *db/db* mice, both skeletal and liver long-chain acylcarnitines were strongly increased compared to lean mice (Figure 2). BAS treatment had no effect on hepatic long-chain acylcarnitine content in db/db mice (Figure 2A). In contrast, in skeletal muscle of *db/db* mice a strong reduction of long-chain

acylcarnitine content was observed which almost reached the level of untreated lean mice (Figure 2B). Specifically saturated long-chain acylcarnitine species of palmitic (C16:0) and stearic acid (C18:0) were affected (Fig. 2C). Apparently, increased metabolic clearance of glucose by peripheral tissue is paralleled by changes in long-chain acylcarnitine content of muscle indicative of increased efficiency of mitochondrial fatty acid oxidation.

DISCUSSION

The leptin receptor deficient *db/db* mouse is a widely utilized mouse model of type 2 diabetes as it displays several of the features of human type 2 diabetes at 12 weeks of age [40]. However, no characterization describing disturbances of *in vivo* hepatic glucose metabolism in this mouse model is available. Here, we first show that leptin receptor deficiency results in a variety of alterations of *in vivo* hepatic glucose metabolism, the most prominent perturbations being the massively increased glucokinase flux and glucose cycling in *db/db* mice. Secondly, we tested whether the bile acid sequestrant Colesevelam (BAS) induces its reported blood glucose-lowering actions [23, 24] by specific alterations of *in vivo* hepatic glucose metabolism. Our results, however, demonstrate that BAS treatment increased metabolic clearance of glucose by peripheral tissue without affecting hepatic glucose production. Unexpectedly, we observed a three-fold induction of hepatic FGF21 gene expression and a decrease in skeletal muscle long-chain acylcarnitine content in treated *db/db* mice. We speculate this lowering in skeletal muscle long-chain acylcarnitines to be indicative of an improved skeletal muscle insulin sensitivity and glucose uptake. Increased hepatic FGF21 gene expression might provide a novel role for FGF21 as modulator of peripheral glucose metabolism upon BAS.

A major and novel observation of this study concerns the disturbances in *in vivo* hepatic glucose metabolism in *db/db* mice. Most strikingly, glucokinase flux was drastically increased in *db/db* mice compared to lean mice. Irrespective the dramatic increase of the HOMA index in *db/db* compared to lean mice, the metabolic clearance of glucose by the liver was essentially not affected. Moreover,

a similar ineffectiveness of insulin to modulate hepatic glucose metabolism was apparent from the data on *de novo* synthesis of glucose-6-phosphate: biosynthesis of glucose-6-phosphate was only slightly decreased (~20%) in *db/db* compared to lean mice. Moreover, fluxes through glucokinase and *de novo* glucose-6-phosphate synthesis were insensitive towards changes in insulin concentration.

Secondly, we studied whole body glucose metabolism. We found that the metabolic clearance rate of glucose by peripheral organs was ~3-fold lower in *db/db* mice at blood glucose concentrations more than 3 times higher and plasma insulin concentrations ~7.5 times higher than those of lean mice. It is of interest to compare the hepatic clearance of glucose by glucokinase with values obtained for peripheral glucose clearance in *db/db* mice. In *db/db* mice, peripheral clearance of glucose was strongly reduced, whereas hepatic clearance of glucose by glucokinase was hardly affected compared to lean mice. Furthermore, the rate of gluconeogenesis was hardly affected by the prevailing high glucose and insulin concentrations in *db/db* mice. Similar observations were made previously by us in *ob/*ob mice [25]. It clearly indicates that the increase in blood glucose concentration in both *db/db* and *ob/ob* mice is driven by an impaired uptake and metabolism of glucose in peripheral organs rather than by increased hepatic glucose production.

Treatment with BAS has been shown to reduce plasma glucose levels in type 2 diabetic humans [41, 42] and rodents [23, 24]. We tested whether the glucose-lowering actions of BAS in *db/db* mice were due to improvement of disturbed hepatic glucose metabolism. Plasma glucose concentration and *de novo* glucose-6-phosphate synthesis decreased upon BAS treatment in *db/db* mice. Furthermore, insulin concentration tended to decrease, albeit not significantly, when compared to untreated *db/db* mice. The glucokinase flux as well as the glucose cycling rate, however, remained invariantly high. Thus, changes in liver glucose metabolism do not mediate the glucose-lowering effect of BAS.

Intriguingly, we also observed an increase in hepatic triglyceride content in BAS treated *db/db* mice, likely due to induction of lipogenesis [24], which is often associated with impaired insulin sensitivity and potentially may have counteracted a BAS induced improvement of hepatic insulin sensitivity. However, the relationship between insulin sensitivity and hepatic triglyceride levels is not

straightforward and fatty liver does not necessarily result in increased insulin resistance. For example, fatty liver induced by an LXR-agonist resulted in improved whole body insulin sensitivity, lowered blood glucose levels and increased metabolic glucose clearance in *ob/ob* mice while massively increasing hepatic triglyceride levels [43]. Moreover, adding treatment of healthy mice with an LXR-agonist induced hepatic steatosis while not affecting blood glucose levels, whole-body insulin sensitivity nor metabolic clearance rate [43].

BAS improved peripheral glucose clearance in *db/db* mice. Interestingly, long-chain acylcarnitine content in *db/db* skeletal muscle, specifically long-chain acylcarnitines of palmitic and stearic acid, decreased upon BAS treatment. Acylcarnitines are by-products of mitochondrial fatty acid oxidation and are formed upon acyl transfer from acyl-CoA to carnitine. Composition and content of acylcarnitines can reflect both high and low rates of mitochondrial fatty acid oxidation. In inborn errors of mitochondrial fatty acid oxidation, in which fatty acid oxidation is impaired, acylcarnitines typically accumulate in tissues and plasma. Additionally, it has been shown that excessive fatty acid oxidation in diabetic mice results in inefficient oxidation. Increased content of long-chain acylcarnitines in skeletal muscle of *db/db* mice compared to lean mice could therefore be indicative of impaired oxidation of long-chain fatty acids in skeletal muscle mitochondria [39]. This results in metabolical inflexibility, *i.e.*, the switch to glucose oxidation cannot be made and insulin is unable to stimulate glucose oxidation [44]. BAS treatment resulted in a clear-cut decrease of long-chain acylcarnitine content of skeletal muscle in *db/db* mice only, nearly to the level that was observed in lean mice. This is suggestive of a more efficient mitochondrial fatty acid oxidation. Concomitantly, a higher metabolic clearance rate of glucose was observed, indicative of an increased ability of the mitochondria to switch to carbohydrate oxidation and indicative of an improved insulin sensitivity.

We also observed an increased ileal expression of glucagon-like-peptide-1 upon BAS treatment (data not shown), which has been associated with an improved insulin sensitivity [45]. Concomitantly, BAS induced a slight malabsorption of dietary fatty acids in lean and *db/db* mice (data not shown), indicative of an increased amount o fatty acids passing through the distal parts of the intestine

upon BAS treatment. Fatty acids can induce GLP-1 by stimulation of L-cells in the ileum [46]. Indeed, very recent work by Shang et al showed that 8 week dietary addition of BAS in insulin-resistant, diet-induced obese rats led to an increased Glp-1 release during an oral glucose tolerance test while plasma glucose and insulin concentrations decreased compared to untreated insulin-resistant diet-induced obese rats [47].

The question arises how BAS treatment, which interrupts the enterohepatic circulation of bile acids, can exert its beneficial effects in peripheral tissues. We observed that BAS induced a 2-fold increase in hepatic FGF21 gene expression in lean and 3-fold increase in *db/db* mice. Overexpression of FGF21 in livers of *db/db* or *ob/ob* mice or administration of recombinant FGF21 to *db/db* or *ob/ob* mice or to diabetic Zucker rats have been shown to have beneficial effects on insulin sensitivity and glucose clearance [36, 48, 49]. In this respect, FGF21 might provide a link to communicate changes in liver metabolism to peripheral tissues to allow for metabolic adaptation. How FGF21 exactly brings about these changes remains, however, elusive at the moment.

In conclusion, this study is the first to characterize hepatic glucose fluxes in *db/db* mice. Glucokinase flux and rates of hepatic glucose output were massively increased in these mice compared to lean mice. Additionally, *db/db* mice had lower metabolic clearance rates of glucose by peripheral tissues compared to lean mice. Decreased plasma glucose levels upon BAS treatment were mainly attributable to increased metabolic clearance of glucose by peripheral tissues: hepatic glucose output remained unaffected. Interestingly, skeletal muscle long-chain acylcarnitine content was decreased in BAS-treated *db/db* mice. Increased hepatic FGF21 gene expression levels might play a crucial role in modulating peripheral glucose handling upon BAS-treatment. This hypothesis, however, requires further investigation.

REFERENCES

1 King, H., Aubert, R. E. and Herman, W. H., Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections, Diabetes Care, 1998, 21: 1414-1431.
2 DeFronzo, R. A., Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview, Diabetologia, 1992, 35: 389-397.

3 Phielix, E. and Mensink, M., Type 2 diabetes mellitus and skeletal muscle metabolic function, Physiol Behav, 2008, 94: 252-258.
4 Corpeleijn, E., Saris, W. H. and Blaak, E. E., Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle, Obes Rev, 2009, 10: 178-193.
5 Donovan, J. M., Von Bergmann, K., Setchell, K. D., Isaacsohn, J., Pappu, A. S., Illingworth, D. R., Olson, T. and Burke, S. K., Effects of colesevelam HC1 on sterol and bile acid excretion in patients with type IIa hypercholesterolemia, Dig Dis Sci, 2005, 50: 1232-1238.
6 Davidson, M. H., The use of colesevelam hydrochloride in the treatment of dyslipidemia: a review, Expert Opinion on Pharmacotherapy, 2007, 8: 2569-2578.
7 Insull, W., Jr., Clinical utility of bile acid sequestrants in the treatment of dyslipidemia: a scientific review, South Med J, 2006, 99: 257-273.
8 Bays, H. E., Goldberg, R. B., Truitt, K. E. and Jones, M. R., Colesevelam hydrochloride therapy in patients with type 2 diabetes mellitus treated with metformin: glucose and lipid effects, Arch Intern Med, 2008, 168: 1975-1983.
9 Fonseca, V. A., Rosenstock, J., Wang, A. C., Truitt, K. E. and Jones, M. R., Colesevelam HCl improves glycemic control and reduces LDL cholesterol in patients with inadequately controlled type 2 diabetes on sulfonylurea-based therapy, Diabetes Care, 2008, 31: 1479-1484.
10 Goldberg, R. B., Fonseca, V. A., Truitt, K. E. and Jones, M. R., Efficacy and safety of colesevelam in patients with type 2 diabetes mellitus and inadequate glycemic control receiving insulin-based therapy, Arch Intern Med, 2008, 168: 1531-1540.
11 Onishi, T., Sano, N. and Takikawa, H., Effect of colestimide on absorption of unconjugated bile acids in the rat jejunum, J Gastroenterol Hepatol, 2002, 17: 697-701.
12 Matsuzaki, Y., Colestimide: the efficacy of a novel anion-exchange resin in cholestatic disorders, J Gastroenterol Hepatol, 2002, 17: 1133-1135.
13 Yamagata, K., Daitoku, H., Shimamoto, Y., Matsuzaki, H., Hirota, K., Ishida, J. and Fukamizu, A., Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1, J Biol Chem, 2004, 279: 23158-23165.
14 Zhang, Y., Lee, F. Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F. J., Willson, T. M. and Edwards, P. A., Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice, Proc Natl Acad Sci U S A, 2006, 103: 1006-1011.
15 De Fabiani, E., Mitro, N., Gilardi, F., Caruso, D., Galli, G. and Crestani, M., Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle, J Biol Chem, 2003, 278: 39124-39132.
16 Cariou, B., Duran-Sandoval, D., Kuipers, F. and Staels, B., Farnesoid X receptor: a new player in glucose metabolism?, Endocrinology, 2005, 146: 981-983.
17 Duran-Sandoval, D., Cariou, B., Fruchart, J. C. and Staels, B., Potential regulatory role of the farnesoid X receptor in the metabolic syndrome, Biochimie, 2005, 87: 93-98.
18 Duran-Sandoval, D., Mautino, G., Martin, G., Percevault, F., Barbier, O., Fruchart, J. C., Kuipers, F. and Staels, B., Glucose regulates the expression of the farnesoid X receptor in liver, Diabetes, 2004, 53: 890-898.
19 Stayrook, K. R., Bramlett, K. S., Savkur, R. S., Ficorilli, J., Cook, T., Christe, M. E., Michael, L. F. and Burris, T. P., Regulation of carbohydrate metabolism by the farnesoid X receptor, Endocrinology, 2005, 146: 984-991.
20 Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burka, L. T., McMorris, T., Lamph, W. W., Evans, R. M. and Weinberger, C., Identification of a nuclear receptor that is activated by farnesol metabolites, Cell, 1995, 81: 687-693.
21 Lefebvre, P., Lien, F., Cariou, B., Kuipers, F. and Staels, B., Role of bile acids and bile acid receptors in metabolic regulation, Physiological reviews, 2009: 147.

22 Cariou, B., van Harmelen, K., Duran-Sandoval, D., van Dijk, T. H., Grefhorst, A., Abdelkarim, M., Caron, S., Torpier, G., Fruchart, J. C., Gonzalez, F. J., Kuipers, F. and Staels, B., The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice, J Biol Chem, 2006, 281: 11039-11049.
23 Kobayashi, M., Ikegami, H., Fujisawa, T., Nojima, K., Kawabata, Y., Noso, S., Babaya, N., Itoi-Babaya, M., Yamaji, K., Hiromine, Y., Shibata, M. and Ogihara, T., Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin, Diabetes, 2007, 56: 239-247.
24 Herrema, H., Meissner, M., van Dijk, T. H., Brufau, G., Boverhof, R., Oosterveer, M. H., Reijngoud, D. J., Muller, M., Stellaard, F., Groen, A. K. and Kuipers, F., Bile salt sequestration induces hepatic de novo lipogenesis through farnesoid X receptor- and liver X receptoralpha-controlled metabolic pathways in mice, Hepatology, 2009.
25 Bandsma, R. H., Grefhorst, A., van Dijk, T. H., van der Sluijs, F. H., Hammer, A., Reijngoud, D. J. and Kuipers, F., Enhanced glucose cycling and suppressed de novo synthesis of glucose-6-phosphate result in a net unchanged hepatic glucose output in ob/ob mice, Diabetologia, 2004, 47: 2022-2031.
26 van Dijk, T. H., van der Sluijs, F. H., Wiegman, C. H., Baller, J. F., Gustafson, L. A., Burger, H. J., Herling, A. W., Kuipers, F., Meijer, A. J. and Reijngoud, D. J., Acute inhibition of hepatic glucose-6-phosphatase does not affect gluconeogenesis but directs gluconeogenic flux toward glycogen in fasted rats. A pharmacological study with the chlorogenic acid derivative S4048, J Biol Chem, 2001, 276: 25727-25735.
27 van Dijk, T. H., Boer, T. S., Havinga, R., Stellaard, F., Kuipers, F. and Reijngoud, D. J., Quantification of hepatic carbohydrate metabolism in conscious mice using serial blood and urine spots, Anal Biochem, 2003, 322: 1-13.
28 Lee, W. N., Byerley, L. O., Bergner, E. A. and Edmond, J., Mass isotopomer analysis: theoretical and practical considerations, Biol Mass Spectrom, 1991, 20: 451-458.
29 Hellerstein, M. K. and Neese, R. A., Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers, Am J Physiol, 1992, 263: E988-1001.
30 Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 1959, 37: 911-917.
31 Chace, D. H., DiPerna, J. C., Mitchell, B. L., Sgroi, B., Hofman, L. F. and Naylor, E. W., Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death, Clin Chem, 2001, 47: 1166-1182.
32 Derks, T. G., Boer, T. S., van Assen, A., Bos, T., Ruiter, J., Waterham, H. R., Niezen-Koning, K. E., Wanders, R. J., Rondeel, J. M., Loeber, J. G., Ten Kate, L. P., Smit, G. P. and Reijngoud, D. J., Neonatal screening for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in The Netherlands: the importance of enzyme analysis to ascertain true MCAD deficiency, J Inherit Metab Dis, 2008, 31: 88-96.
33 Gates, S. C. and Sweeley, C. C., Quantitative metabolic profiling based on gas chromatography, Clin Chem, 1978, 24: 1663-1673.
34 Plosch, T., Kok, T., Bloks, V. W., Smit, M. J., Havinga, R., Chimini, G., Groen, A. K. and Kuipers, F., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1, J Biol Chem, 2002, 277: 33870-33877.
35 Lefever, S., Vandesompele, J., Speleman, F. and Pattyn, F., RTPrimerDB: the portal for real-time PCR primers and probes, Nucleic Acids Res, 2009, 37: D942-945.
36 Kharitonenkov, A., Shiyanova, T. L., Koester, A., Ford, A. M., Micanovic, R., Galbreath, E. J., Sandusky, G. E., Hammond, L. J., Moyers, J. S., Owens, R. A., Gromada, J., Brozinick, J. T., Hawkins, E. D., Wroblewski, V. J., Li, D. S., Mehrbod, F., Jaskunas, S. R. and Shanafelt, A. B., FGF-21 as a novel metabolic regulator, J Clin Invest, 2005, 115: 1627-1635.
37 Chavez, J. A. and Summers, S. A., Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1

adipocytes and C2C12 myotubes, Archives of Biochemistry and Biophysics, 2003, 419: 101-109.
38 Ragheb, R., Shanab, G. M. L., Medhat, A. M., Seoudi, D. M., Adeli, K. and Fantus, I. G., Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: Evidence for PKC activation and oxidative stress-activated signaling pathways, Biochemical and Biophysical Research Communications, 2009, 389: 211-216.
39 Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D. and Muoio, D. M., Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance, Cell Metab, 2008, 7: 45-56.
40 Shafrir, E., Animal models of non-insulin-dependent diabetes, Diabetes Metab Rev, 1992, 8: 179-208.
41 Garg, A. and Grundy, S. M., Cholestyramine therapy for dyslipidemia in non-insulin-dependent diabetes mellitus. A short-term, double-blind, crossover trial, Ann Intern Med, 1994, 121: 416-422.
42 Zieve, F. J., Kalin, M. F., Schwartz, S. L., Jones, M. R. and Bailey, W. L., Results of the glucose-lowering effect of WelChol study (GLOWS): a randomized, double-blind, placebo-controlled pilot study evaluating the effect of colesevelam hydrochloride on glycemic control in subjects with type 2 diabetes, Clin Ther, 2007, 29: 74-83.
43 Grefhorst, A., van Dijk, T. H., Hammer, A., van der Sluijs, F. H., Havinga, R., Havekes, L. M., Romijn, J. A., Groot, P. H., Reijngoud, D.-J. and Kuipers, F., Differential effects of pharmacological liver X receptor activation on hepatic and peripheral insulin sensitivity in lean and ob/ob mice, Am J Physiol Endocrinol Metab, 2005, 289: E829-838.
44 Boden, G., Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes, Exp Clin Endocrinol Diabetes, 2003, 111: 121-124.
45 Freeman, J. S., Role of the incretin pathway in the pathogenesis of type 2 diabetes mellitus, Cleve Clin J Med, 2009, 76 Suppl 5: S12-19.
46 Rocca, A. S., LaGreca, J., Kalitsky, J. and Brubaker, P. L., Monounsaturated Fatty Acid Diets Improve Glycemic Tolerance through Increased Secretion of Glucagon-Like Peptide-1, Endocrinology, 2001, 142: 1148-1155.
47 Shang, Q., Saumoy, M., Holst, J. J., Salen, G. and Xu, G., Colesevelam improves insulin resistance in a diet-induced obesity (F-DIO) rat model by increasing the release of GLP-1, Am J Physiol Gastrointest Liver Physiol, 298: G419-424.
48 Coskun, T., Bina, H. A., Schneider, M. A., Dunbar, J. D., Hu, C. C., Chen, Y., Moller, D. E. and Kharitonenkov, A., Fibroblast growth factor 21 corrects obesity in mice, Endocrinology, 2008, 149: 6018-6027.
49 Xu, J., Lloyd, D. J., Hale, C., Stanislaus, S., Chen, M., Sivits, G., Vonderfecht, S., Hecht, R., Li, Y. S., Lindberg, R. A., Chen, J. L., Jung, D. Y., Zhang, Z., Ko, H. J., Kim, J. K. and Veniant, M. M., Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice, Diabetes, 2009, 58: 250-259.

CHAPTER 4

Exercise enhances whole-body cholesterol turnover in mice

M. Meissner, R. Havinga, R. Boverhof, I. Kema, A. K. Groen, F. Kuipers

Med Sci Sports Exerc. 2010, 42(8):1460-8

ABSTRACT

Purpose Regular exercise reduces cardiovascular risk in humans by reducing cholesterol levels but underlying mechanisms have not been fully explored. Exercise might provoke changes in cholesterol and bile acid metabolism and thereby reduce cardiovascular risk. We examined whether voluntary wheel running in mice modulates cholesterol and bile acid metabolism.

Methods Male mice (10 wk old) were randomly assigned to have access to a voluntary running wheel for 2 wks (RUN) or remained sedentary (SED). Running wheel activity was recorded daily. In a first experiment, fecal sterol outputs, fecal bile acid profiles, plasma parameters and expression levels of genes involved in cholesterol and bile acid metabolism were determined. In a second experiment, bile flow, biliary bile acid profile and biliary secretion rates of cholesterol, phopsholipids and bile acids were determined.

Results RUN ran an average of 10 km per day and displayed lower plasma cholesterol compared to SED ($p=0.030$). Fecal bile acid loss was induced by ~30% in running mice compared to SED ($p=0.001$). A ~30% increase in fecal cholesterol output in RUN ($p=0.014$) was consistent with changes in parameters of cholesterol absorption, like reduced plasma plant sterol / cholesterol ratio ($p=0.044$) and decreased jejunal expression of Npc1l1 ($p=0.013$). Supportive of an increased cholesterol synthesis to compensate for fecal sterol loss were increased hepatic mRNA levels of Hmgcr ($p=0.006$) and an increased plasma lathosterol/cholesterol ratio ($p=0.001$) in RUN.

Conclusion Voluntary wheel running increased cholesterol turnover in healthy mice due to an increased fecal bile acid excretion and a decreased intestinal cholesterol absorption. Enhanced cholesterol turnover may contribute to the established reduction of cardiovascular risk induced by regular exercise.

INTRODUCTION

Exercise is known as a preventative and therapeutic for cardiovascular diseases [1] but exactly how exercise exerts its beneficial actions on cardiovascular risk is largely unknown. One of the major hallmarks in exercise-induced risk reduction observed in human studies is an improvement in plasma lipid levels [2, 3]. Specifically, high density lipoprotein levels increase with exercise and concomitantly total cholesterol, low-density lipoprotein cholesterol and triglycerides decrease [2, 3]. Yet, the mechanisms behind such exercise-induced improvements in plasma lipids have not been defined. An enhanced transport of peripheral cholesterol towards the liver for subsequent secretion as free cholesterol or bile acids into bile and eventual excretion into feces has been suggested [4, 5]. In fact this pathway, known as the reverse cholesterol transport (RCT), constitutes the major elimination route for excess cholesterol from the body [6]. HDL is considered to be the carrier of cholesterol in the RCT pathway. To date, the effects of exercise on this pathway are poorly understood [4, 5]. However, if exercise enhances RCT this should be reflected in an enhanced turnover of cholesterol and, possibly, modulations in bile acid metabolism.

Excess cholesterol can be removed from the body either as such or after conversion into bile acids. The liver secretes free cholesterol into bile, which is eventually released into the intestine where it mixes with dietary cholesterol. Fractional absorption of cholesterol in humans shows a range between 30-70%. While in mice values of 30-50% are reported [7]. Bile acids are synthesized from cholesterol exclusively in the liver and expelled into the intestinal lumen after ingestion of a meal. Bile acids are important molecules for the emulsification and reabsorption of fats in the intestine [8]. The majority of bile acids are reabsorbed from the terminal ileum, transported back to the liver for resecretion into bile (enterohepatic circulation). The fraction of bile acids that escapes reabsorption is lost in feces and constitutes an important part of cholesterol turnover, since fecal bile acid loss is compensated for by *de novo* synthesis from cholesterol to maintain the bile acid pool size [9, 10]. This means that pertubations provoking an increased fecal bile acid loss also provoke an increased hepatic *de novo* bile acid synthesis.

Indeed, past limited work indicates that exercise potentially modulates cholesterol and bile acid metabolism [11-13]. Early studies on female rats showed that six weeks of voluntary wheel running promoted an increased biliary bile acid, cholesterol and phospholipid secretion [12] and either increased or had no effect on bile flow [12, 13]. Moreover, a recent study in mice showed that 12 weeks of forced treadmill exercise prevents gallstone formation in gallstone-prone mice [11]. Gallstone disease is characterized by an abnormally high biliary cholesterol-to-bile acid and phospholipid ratio [8].

However, these limited data do not provide a clear understanding of the effects of exercise on bile acid and cholesterol turnover in rodents. Therefore the purpose of this study was to clarify whether exercise modulates cholesterol and bile acid turnover in mice. If exercise indeed has such actions, this would offer novel insight in mechanisms contributing to the beneficial effects of exercise on cardiovascular diseases.

METHODS AND MATERIALS

All experiments were approved by the Animal Care and Use Committee of the University of Groningen, The Netherlands. The University of Groningen is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (*Guide for the Care and Use of Laboratory Animals.* In: Council NR, ed. Washington (DC): National Academy Press 1996) and the study was in adherence to ACSM animal care standards.

Animals and voluntary cage-wheel exercise

Ten week old male C57BL/6 mice were purchased from Charles River Laboratories (L'Arbresle, France). Upon arrival at the animal facility, mice were singly housed in a cage (47 x 26 x 14.5 cm^3) in a temperature-controlled room with

a 12:12 light-dark cycle. Throughout the study mice had access to standard commercial pelleted laboratory chow (RMH-B, Hope Farms bv Woerden, the Netherlands) and water *ad libitum*. At 11 weeks of age, mice were randomly selected to either voluntary cage wheel running (RUN) or to remain sedentary (SED). The voluntary running wheel set-up utilized has previously been described [14]. Briefly, the cage of RUN mice was equipped with a hamster-sized metal cage wheel with a diameter of 11 cm which was fitted with a cycle computer containing a digital magnetic counter (Art NO.: K-13-TL SET-P3-NL, Xiron, Netherlands). Each morning total distances ran, total times ran, average and maximum daily speeds were recorded. Twice a week, mice were weighed and food intake was recorded. Exposing mice to a voluntary running wheel for 2 weeks has previously been shown to result in cardiac and skeletal muscle adaptations consistent with those of endurance exercise [14]. Moreover, the voluntary cage wheel set-up provides an anti-stress intervention [15] compared to force exercise interventions which have been found to cause chronic stress-like changes in the hypothalamic–pituitary–adrenal axis [16].

Experimental procedures

There were two primary experiments examining the effect of voluntary cage wheel running on cholesterol and bile acid metabolism: one on fecal, plasma, hepatic and intestinal parameters, and the other on biliary parameters. The endpoint of both experiments was reached after 2 weeks of RUN or SED, at 13 weeks of age.

Experiment 1: Determination of plasma, fecal, hepatic and intestinal parameters of cholesterol and bile acid metabolism

In experiment 1, RUN (n=8) and SED (n=6) mice were sacrificed by heart puncture under isoflurane anesthesia from 06:30-08:30 h. Plasma was stored at -20°C until analysis. The liver was quickly removed, weighed and snap-frozen in liquid nitrogen. The small intestine was excised, flushed with ice cold (4°C) PBS, divided into three sections of equal lengths and subsequently snap-frozen in liquid

nitrogen. Both liver and intestine were stored at -80ºC for later biochemical analysis and RNA isolation.

Plasma and liver lipid analysis

Plasma was collected by centrifugation of blood samples obtained via heart puncture. Plasma total cholesterol, free cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially available assay kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Hepatic lipids were determined after extraction according to Bligh and Dyer [17] using the same commercially available kits as for plasma lipids.

We analyzed plasma plant sterols and lathosterol levels relative to plasma cholesterol levels as makers of intestinal cholesterol absorption (plant sterols) and cholesterol synthesis (lathosterol). The plasma plant sterol (campesterol + sitosterol) / cholesterol ratio is often utilized as a marker for intestinal cholesterol absorption as it correlates very well with rates of fractional intestinal cholesterol absorption [18]. The plasma lathosterol / cholesterol ratio is a marker of cholesterol synthesis [18], because lathosterol is a precursor of the cholesterol biosynthetic pathway. Plasma plant sterol (campesterol and sitosterol) and lathosterol concentrations were determined by gas chromatography, as described by Windler et al.[19]. Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography on a Superose 6 column using Akta Purifier (GE Healthcare, Diegem, Belgium).

Fecal Parameters

Forty-eight-hour feces productions were collected before running wheel exposure and at 2 weeks of running wheel exposure. Feces were dried, weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total bile acids by an enzymatic fluorimetric assay [20]. Neutral sterols and bile acid profiles were determined according to Arca et al. [21] and Setchell et al. [22], respectively.

Supplemental Table 1: Progression in Running Wheel Activity. Values are means ± SD for RUN (n=8) over the two week running wheel intervention

	DAY 1	***Day 7***	***Day 14***
Running Wheel Activity			
Average daily distance (km)	5.35 ± 1.7	7.88 ± 1,7	10.2 ± 2.2
Maximum Speed ($km \times h^{-1}$)	2.83 ± 0.6	2.94 ± 0.23	3.1 ± 0.16
Average speed ($km \times h^{-1}$)	0.87 ± 0.10	1.34 ± 0.14	1.78 ± 0.18
Average daily time ran (h:min)	6:05 ± 1:32	5:54 ± 0:55	5:46 ± 0:52

RNA isolation and PCR procedures

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described by Plösch and coworkers [23]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96-well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been deposited at the RTprimerDB [24]. PCR results were normalized to β-actin.

Experiment 2: Determination of biliary parameters of cholesterol and bile acid metabolism

Another eight RUN and five SED mice underwent a gallbladder cannulation for collection of bile at 13 weeks of age [23]. Briefly, mice were anaesthetized by intraperitoneal injection with Hypnorm ® (1 $ml \cdot kg^{-1}$) and diazepam (10 $mg \cdot kg^{-1}$). During the 30-min bile collection period, mice were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1 $g \cdot ml^{-1}$ for bile. Bile was stored at -20°C until analysis. Total biliary bile acids were determined by an enzymatic fluorimetric assay [20]. Levels of biliary cholesterol and phospholipids were measured as

described by Kuipers et al. [25]. Biliary bile acid composition was determined as described by Hulzebos et al. [26].

Statistics

Statistical analysis was assessed using the Mann-Whitney-U-Test (SPSS 12.0.1 for Windows). All data are expressed as means ± SD. A *P*-value of <0.05 was accepted as statistically significant.

Table 1. Biometrical parameters and running wheel data. Values are means ± SD at two weeks of running for SED (n=6) and RUN (n=8); *p<0.05 vs. SED

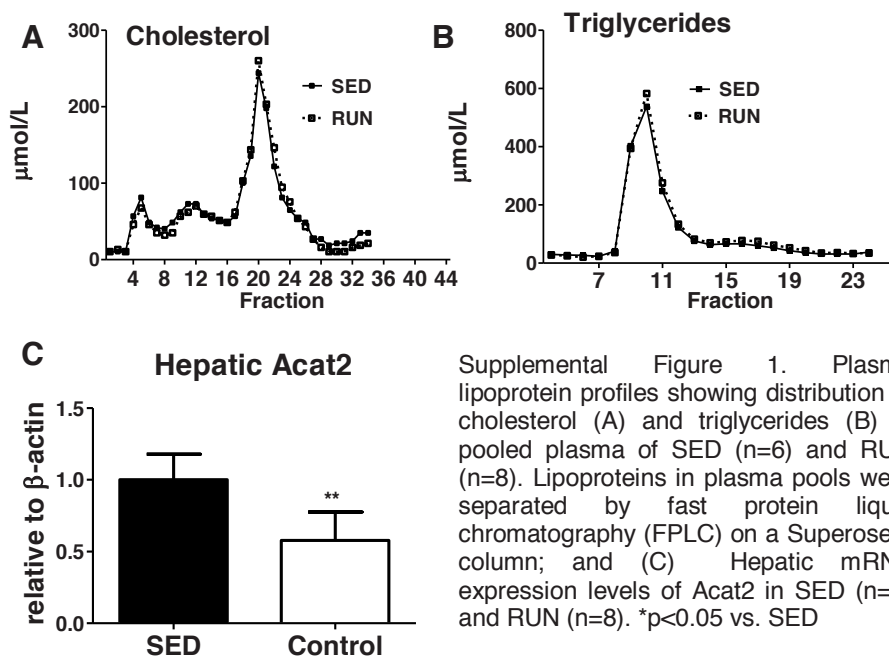
	SED	***RUN***
Biometrical Parameters		
Bodyweight (g)	26.5 ± 0.6	25.5 ± 1.0
Food intake (g)	4.1 ± 0.3	5.4 ± 0.5*
Liverweight (g)	1.43 ± 0.16	1.40 ± 0.07
Liverweight / Bodyweight (%)	5.5 ± 0.2	5.4 ± 0.3
Small Intestinal Length (cm)	33.8 ± 1.5	33.5 ± 1.5
Running Wheel Activity		
Average daily distance (km)	ND	10.2 ± 2.2
Average speed (km/h)	ND	1.78 ± 0.18
Average daily time ran (h:min)	ND	5:46 ± 0:52

RESULTS

Basal parameters upon two weeks of voluntary wheel exercise

Exposing 11-week old chow fed C57BL/6 mice to a voluntary running wheel for two weeks did not affect body weight, liver weight, body weight / liver weight ratio nor intestinal length compared to sedentary mice (Table 1). Mice exposed to a running wheel averaged a daily distance of ~10 km (Table 1) and were progressively running at a greater average speed and for a longer time and distance during the 2 week running wheel exposure (Supplement Table 1).

Running mice consumed ~30% more food than sedentary mice did (Table 1). Running mice had decreased total and free plasma cholesterol levels compared to sedentary controls (Table 2), but plasma lipoprotein profiles for cholesterol or triglycerides were not different between running and sedentary mice (Supplemental Figure 1). Hepatic total and free cholesterol store were not affected by the running intervention (Table 2). It seems that there is a small, albeit not significant, effect of running in decreasing hepatic esterified cholesterol content coinciding with a decrease in hepatic mRNA expression levels of Acat2 (Supplemental Figure 1), the major cholesterol esterifying enzyme. In addition, running mice displayed approximately 20% lower hepatic triglyceride content compared to sedentary controls (Table 2).



Supplemental Figure 1. Plasma lipoprotein profiles showing distribution of cholesterol (A) and triglycerides (B) in pooled plasma of SED (n=6) and RUN (n=8). Lipoproteins in plasma pools were separated by fast protein liquid chromatography (FPLC) on a Superose 6 column; and (C) Hepatic mRNA expression levels of Acat2 in SED (n=6) and RUN (n=8). *p<0.05 vs. SED

Voluntary wheel exercise increased fecal neutral sterol and bile acid output

To determine whether 2 weeks of voluntary running wheel exercise alters cholesterol and bile acid metabolism in healthy chow-fed mice, we first assessed fecal parameters of cholesterol and bile acid metabolism. For this study, feces was

Table 2. Plasma and liver lipids. Values are means ± SD. at two weeks of running for SED (n=6) and RUN (n=8); *p<0.05 vs. SED, **p<0.01 vs. SED

	SED	RUN
Plasma Lipids (mmol·L^{-1})		
Triglycerides	1.2 ± 0.4	1.1 ± 0.3
Total Cholesterol	2.4± 0.3	2.1 ± 0.2*
Free Cholesterol	1.2 ± 0.2	0.9 ± 0.2*
Cholesterol Esters	1.2 ± 0.2	1.1 ± 0.2
Liver Lipids (nmol·mg liver^{-1})		
Triglycerides	29.0 ± 5.6	20.7± 4.1**
Total Cholesterol	6.1 ± 1.3	5.3 ± 0.6
Free Cholesterol	5.3 ± 1.5	4.7 ± 0.6
Cholesterol Esters	0.8 ± 0.1	0.7 ± 0.1

collected during the last 48 hrs of the intervention in running and control mice to determine the fecal neutral sterol and bile acid output, as well as the fecal bile acid profile. We found that running mice had a significantly increased fecal production, fecal neutral sterol and fecal bile output (all by ~30%) compared to sedentary controls (Figures 1A-C), while no differences in any of these parameters was observed between RUN and SED before the intervention (data not shown).. Secondly, as shown in Table 3A, running mice had an increased fecal deoxycholate output compared to control mice. Deoxycholate is a secondary bile acid species which is formed from cholate in the intestine. The increasein fecal deoxycholate output in runners was paralleled by a non-significant decrease in fecal cholate output (p=0.053). Thus, the total cholate output, which is the sum of cholate and deoxycholate, remained unaltered (Table 3A). Since under steady state conditions fecal bile acid loss equals hepatic synthesis rate, these results imply that running wheel activity accelerates the conversion of cholesterol to bile acids in the liver.

Voluntary running wheel exercise increases biliary bile acid secretion

Observing this running-induced increase in fecal bile acid excretion, we next asked

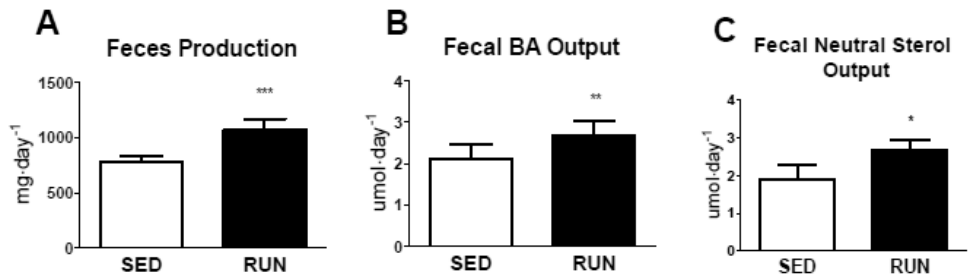


Figure 1. Fecal parameters for SED (n=6) and RUN (n=8) after two weeks of running: A. Daily feces production, B. Daily fecal bile acid output, C. Daily fecal neutral sterol output. *p<0.05 vs. SED, **p<.0.01 vs. SED, ***p<0.001 vs. SED

whether this increased loss was reflected in changes in biliary parameters. Thus, we subjected another set of mice of both groups to gallbladder cannulations for the collection of hepatic bile to examine the effect of voluntary running on bile flow, biliary bile acid, cholesterol and phospholipid secretion. We found a trend for a higher bile flow in running mice (*Figure 2A*). Then, as shown in Figure 2B, running mice had an increased biliary total bile acid secretion (Figure 2B) and also an increased absolute biliary bile acid concentration (27.78 ± 2.31 and 33.40 ± 4.52 $mmol \cdot L^{-1}$, control and running mice respectively, p=0.042). However, secretion rates of biliary cholesterol and phospholipids were not affected by 2 weeks of voluntary wheel running (Figure

2C and D). Next, running increased the biliary total cholate-derived bile acid output because of an increase in biliary cholate output while biliary deoxycholate remained unchanged (Table 3B). This increase in total cholates was reflected in an increased biliary cholate / chenodeoxycholate ratio upon running. Thus, the running-induced increase in fecal bile acid loss was paralleled by changes in biliary bile acid parameters.

We found that the running-induced fecal bile acid loss coincided with changes of some genes, but not others, implicated in bile acid transport in liver and intestine. For example, no changes were observed in ileal mRNA expression levels of Asbt (Slc10a2), which functions in active absorption of bile acids from the intestinal lumen

into the ileal enterocytes (Figure 3A) nor Fgf15 (Figure 3B), which is induced by bile acids in the ileum and represses bile acid synthesis *via* signaling mechanisms. Running mice displayed lower intestinal mRNA expressions of Ostα (Figure 3C) and Ostβ (Figure 3D), which act together in basolateral efflux of bile acids from the ileum into the portal blood. Moreover, the hepatic mRNA expression levels of Ntcp (Slc10a1) (Figure 3E), a basolateral bile acid transporter acting to clear bile acids from the portal blood into the hepatocyte, was also decreased in running mice. On the other hand, mRNA expression levels of Bsep (Abcb11) (Figure 3F), a

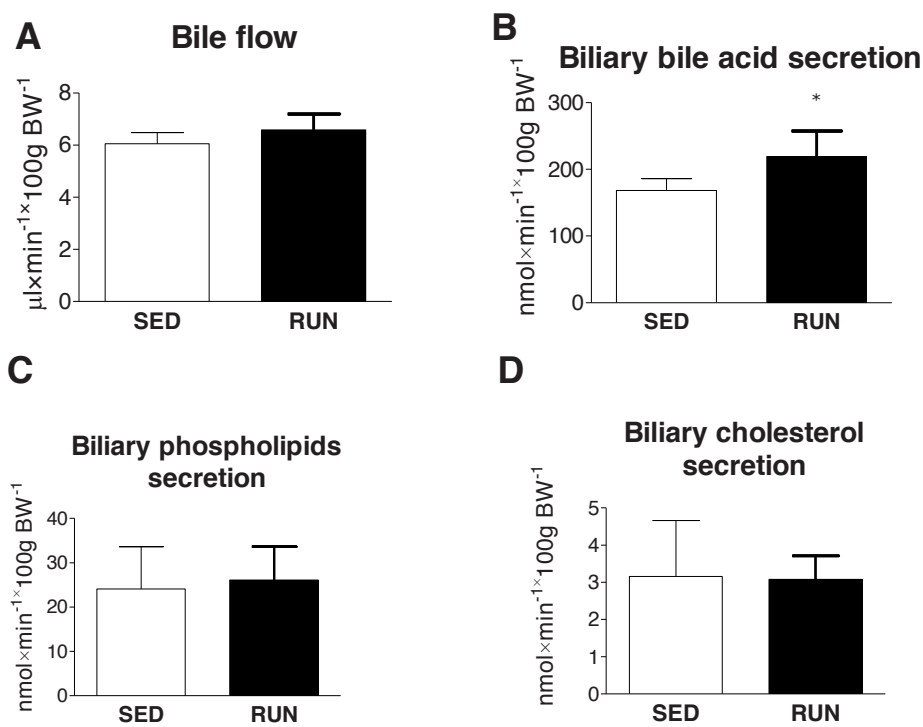


Figure 2. Biliary Parameters for SED (n=5) and RUN (n=8) after 2 weeks of running: A. Bile flow, B. Biliary bile acid output. C.Biliary cholesterol output. D. Biliary phospholipid output; $^{*}p<0.05$ vs. SED

acting to transport bile acids from the hepatocyte into the bile, were not affected through the running intervention.

Voluntary running wheel exercise decreases cholesterol absorption and increases cholesterol synthesis

An increased neutral sterol loss is suggestive of a decreased reabsorption and an increased synthesis to compromise the consequences of the loss. We therefore questioned whether the increased fecal bile acid and neutral sterol loss was reflected by changes in markers of cholesterol absorption and synthesis. Indeed, the jejunal mRNA expression of Npc1l1, a protein required for intestinal cholesterol absorption [27], was decreased in running mice (Figure 4A), while the jejunal mRNA expressions of the ATP-binding cassette transporters Abcg5 and Abcg8, two proteins implicated in control of cholesterol absorption [28] [29] were unchanged (Figure 4B and C). Similarly to the reduced Npc1l1 mRNA expression, the plasma total plant sterol / cholesterol ratio, a marker of cholesterol absorption, was

Table 3. Fecal and biliary bile acid composition as percentage of total bile acids. Values are means ± SD at two weeks of running for SED (n=5) and RUN (n=8); CDCA = chenodeoxycholate, *p<0.05 vs. SED

Percentages of Total Bile Acids	SED	RUN
(A) Fecal bile acid profile		
Cholate	31.1 ± 16.8	14.2 ± 2.9
Deoxycholate	29.4 ± 6.6	42.7 ± 5.1*
Total Cholate-derived bile acids	60.5 ± 10.3	56.9 ± 4.7
Chenodeoxycholate	1.6 ± 0.9	0.7 ± 0.6
ß-muricholic acid	6.3 ± 1.3	5.6± 1.6
w-muricholic acid	27.7 ± 9.6	32.3 ± 3.8
a-muricholic acid	3.9 ± 0.5	4.6 ± 0.4*
Total CDCA-derived bile acids	39.5 ± 10.3	43.2 ± 4.7
(B) Biliary bile acid profile		
Cholate	51.3 ± 2.3	57.6 ± 5.3*
Deoxycholate	4.1 ± 1.0	2.9 ± 0.8
Total Cholate-derived bile acids	55.4 ± 2.7	60.5 ± 5.7*
Chenodeoxycholate	2.6 ± 0.6	1.9 ± 0.8
ß-muricholic acid	23.3 ± 4.2	22.1 ± 4.6
w-muricholic acid	12.9 ± 2.6	11.1 ± 2.4
a-muricholic acid	5.8 ± 0.6	4.5 ± 0.5
Total CDCA-derived bile acids	44.6 ± 2.7	39.5 ± 5.7
Cholates / Chenodeoxycholates	1.3 ± 0.1	1.6 ± 0.4*

decreased in running mice (Figure 4E), thus indicating a decreased cholesterol absorption in runners. In addition, we also observed changes in markers of cholesterol synthesis indicative of an increased cholesterol synthesis upon running. This is shown first in the running- induced increase in hepatic mRNA expression levels of Hmgcr, a gene that encodes the rate-controlling enzyme in cholesterol synthesis (Figure 5A), and second in the ~ 60% increase in the plasma lathosterol / cholesterol ratio in running mice (Figure 5B), supportive of an increased whole-body cholesterol synthesis upon running.

DISCUSSION

This study was designed to assess whether voluntary wheel running modulates cholesterol and bile acid metabolism in healthy mice. The experiments presented here show that running modulates a variety of parameters of cholesterol and bile acid metabolism in mice and that these modulations result in an increased cholesterol turnover due to a decreased intestinal cholesterol and bile acid absorption. This has been demonstrated in three ways. First, the finding that running mice increased their fecal neutral sterol and bile acid output is consistent with a variety of parameters reflective of a decreased cholesterol absorption, the decreased plasma total plant
sterol / cholesterol ratio (Figure 4E), which correlates with the rate of cholesterol absorption [18] and the decreased jejunal expression of Npc1l1 (Figure 4A), which is a protein required for intestinal cholesterol absorption. Second, the present data indicate that the running-induced fecal neutral sterol loss translated into an increased cholesterol synthesis (Figure 5A and B), representing a physiological response to an enhanced cholesterol loss. Third, the increase in fecal bile acid output upon running demonstrates an increased *de novo* bile acid synthesis, which contributes to cholesterol turnover.

Our observations of a running-induced increase in fecal production [30], fecal neutral sterol and bile acid output [31] on one hand confirm some of the limited previous work on humans [30-32] but contradict others showing that four months of

training decreased the total fecal neutral sterols excretion in men [32], and that the total fecal bile acid secretion in male distance runners was lower due to an increased fecal production [30]. The relevance of these findings is, however, difficult to evaluate because all previous studies were restricted to middle aged male long distance runners [30-32], were not controlled for dietary intake, age [30-32] or

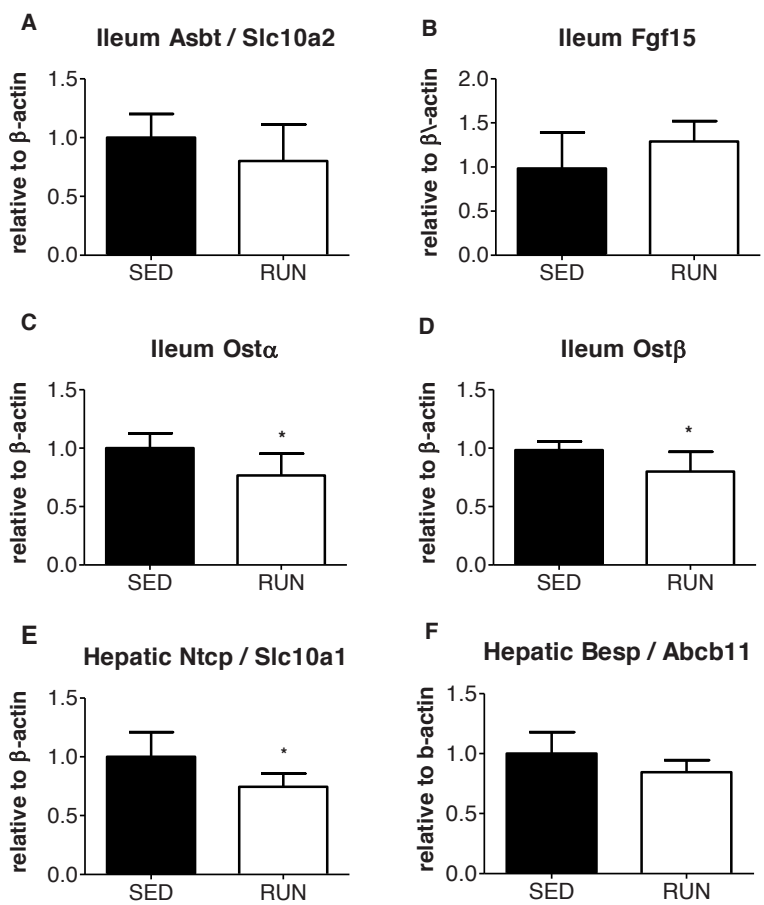


Figure 3. Quantitative RT-qPCR of RNA from intestinal (A through D) and hepatic (E and F) bile acid transporters and signaling molecules in SED (n=6) and RUN (n=8) after two weeks of running. A. Ileal apical sodium dependent bile acid transporter, Asbt / Slc10a2; B. Ileal fibroblast growth factor 15, Fgf15; C and D. Ileal organic solute transporter alpha, Ostα (C) and beta, Ostβ (D); (E) Hepatic sodium-dependent taurocholic cotransporting polypeptide, Ntcp / Slc10a1 and (F) Hepatic bile acid export pump, Bsep / Abcb11; *P<0.05 vs. SED

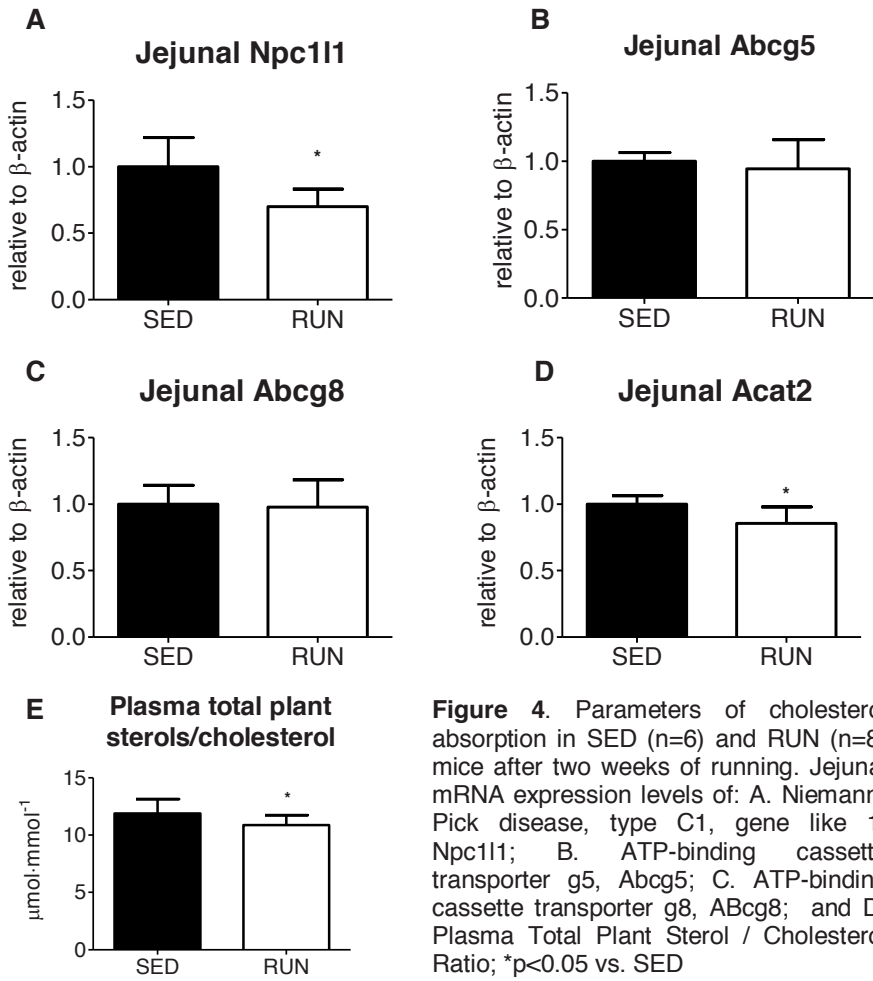


Figure 4. Parameters of cholesterol absorption in SED (n=6) and RUN (n=8) mice after two weeks of running. Jejunal mRNA expression levels of: A. Niemann-Pick disease, type C1, gene like 1, Npc1l1; B. ATP-binding cassette transporter g5, Abcg5; C. ATP-binding cassette transporter g8, ABcg8; and D. Plasma Total Plant Sterol / Cholesterol Ratio; *p<0.05 vs. SED

endurance-training [30, 31], while the present investigation concerns a controlled study of previously sedentary but healthy mice having *at libitum* but monitored access to standard laboratory chow and were exposed to a voluntary running wheel for 2 weeks.

In our study the loss of bile acids is specific for deoxycholate, a secondary bile acid (Table 3 A). Deoxycholate is formed by conversion of cholate, a primary bile acid, by intestinal flora [33]. This increase in fecal deoxycholate is probably due to a decreased bile acid reabsorption in the terminal ileum, thus allowing increased

conversion by intestinal microbiota but also possibly by running-induced alterations in intestinal microbiota leading to an increased production of deoxycholate. Intriguingly, it has recently been shown that male rats exposed to a voluntary wheel running wheel for 5 weeks displayed markedly changed composition of cecal microbiota [34]. However, how exactly voluntary wheel running acts in provoking the specific increase in deoxycholate excretion in mice and what role the role of intestinal microbiota is herein was not the scope of this study but deserves future investigation.

In addition to intestine-modulated changes in fecal bile acid composition, voluntary wheel running affects the intestine further by decreasing jejunal mRNA expression of Npc1l1, a protein critical for intestinal cholesterol absorption [27]. Similarly, we found a running-induced decrease in jejunal acetyl-CoA:cholesterol acetyltransferase 2 (Acat2) mRNA expression levels (Figure 4D). After cholesterol is taken up via Npc1l1 from the intestinal lumen to the enterocyte, it is esterified by Acat2 for proper chylomicron formation. The effect of voluntary running on decreasing intestinal cholesterol absorption was specific for the uptake transporter Npc1l1, as we show here that running did not affect mRNA levels of the cholesterol efflux transporters Abcg5 and Abcg8 in the intestine. Abcg 5 and Abcg8 function as a heterodimer and are responsible for sterol efflux from the enterocytes into the lumen, whereas Npc1l1 is involved in sterol entry from the lumen into the enterocyte [35]. In support of a decreased cholesterol absorption despite no effects on Abcg5/8 expression, a previous study showed that reductions in cholesterol absorption correlated with decreased Npc1l1 mRNA expression while Abcg5/8 mRNA expression remained unchanged [36].

Next, our study indicates in two ways that the exercise-induced malabsorption of sterols is compensated by an increased hepatic cholesterol synthesis. First, running mice had an increased hepatic mRNA expression level of Hmgcr, the rate-limiting enzyme of cholesterol synthesis. And second, running mice had an increased plasma lathosterol / cholesterol ratio. Lathosterol is a precursor of cholesterol synthesis, and an increased lathosterol / cholesterol ratio is indicative of an increased cholesterol synthesis [18]. However, our present results

on young, healthy mice are in discrepancy with previous reports [37, 38]. The inconsistent and limited information that is available regarding the effects of exercise on cholesterol synthesis and absorption is largely restricted to plasma markers of cholesterol synthesis and absorption [37, 38]. These few previous investigations showed either only increases in plasma

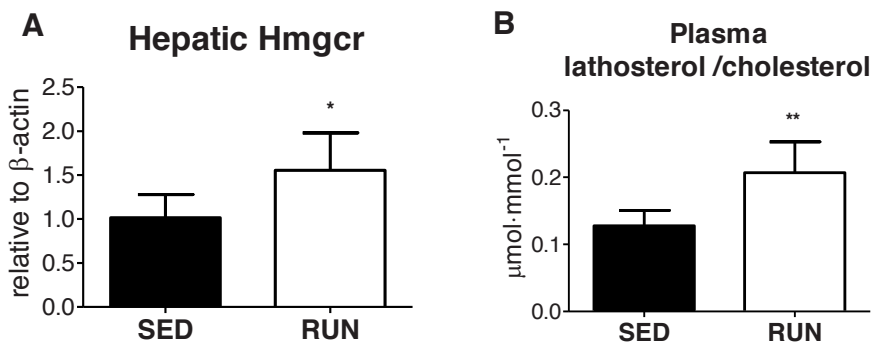
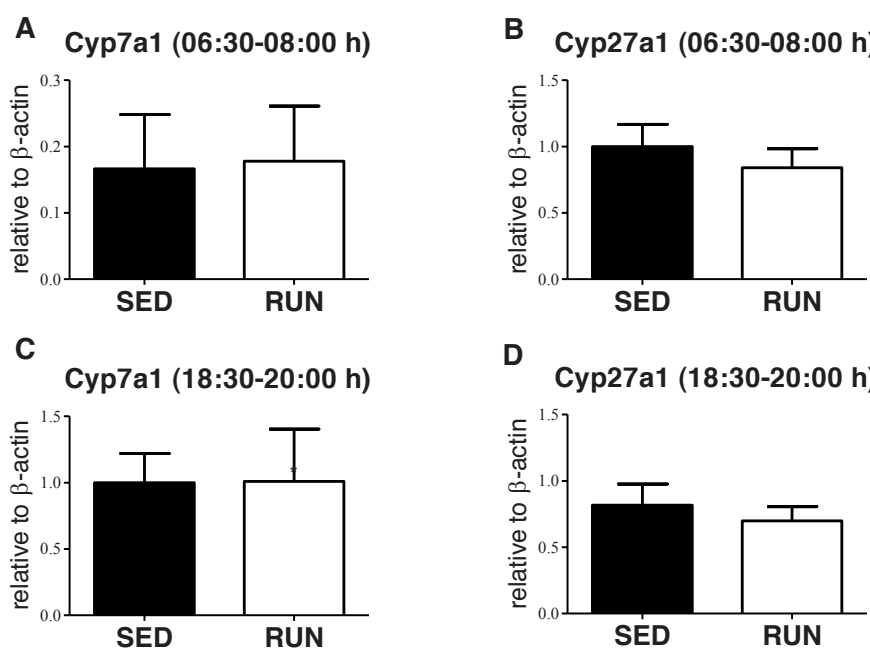


Figure 5. Parameters of cholesterol metabolism in SED (n=6) and RUN (n=8) mice after two weeks of running. A. Hepatic mRNA expression of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, Hmgcr as a marker of cholesterol synthesis B. Plasma lathosterol / cholesterol ratio as a marker of cholesterol; *p<0.05 vs. SED, **p<.0.01 vs. SED

lathosterol / cholesterol [38], or only increases in plasma plant sterols / cholesterol [37] or no effect on markers of cholesterol synthesis or absorption at all [38]. These discrepancies in markers of cholesterol absorption and synthesis between our study and the previous works might be explained by differences in populations studied and exercise utilized. For example, the previous works are limited to humans with lipoprotein abnormalities exercising three times a week for eight weeks or six months [37, 38] while we utilized healthy mice that exercised every day for 2 weeks.

Although our results indicate that voluntary wheel running increases bile acid synthesis, a surprising finding was that expression of the main genes involved in bile acid synthesis, Cyp7a1 and Cyp27a1 (Supplemental Fig 2 A and B), were not affected by the intervention. Genes involved in bile acid synthesis follow a circadian rhythm [39] . For this reason, we asked whether the time for termination

and tissue collection originally chosen did not allow for an observation of differences in gene expression levels. However, we did not observe the anticipated running-induced increase in genes involved in bile acid synthesis when terminating another cohort of running mice and controls between 18:30 and 20:00 hours (12 hours later than all other mice described in this study) (Supplemental Fig. 2 C and D). Thus, within this study the increase in bile acid synthesis upon voluntary wheel running as demonstrated by fecal bile acid loss did not result in an upregulation of mRNA expression levels of key genes involved in bile acid synthesis, specifically Cyp7a1. Possibly a small decrease in reabsorbed intestinal bile acids is sufficient to repress the inhibitory signaling on Cyp7a1 without affecting mRNA expression levels in the voluntary wheel running intervention. In support of this, previous investigations on bile acid synthesis have shown that modulations of bile acid synthesis do not always correlate to changes in Cyp7a1 mRNA expression [26, 40].



Supplemental Figure 2. Quantitative RT-qPCR of liver RNA from SED (n=6) and RUN (n=8) mice terminated between 06:30-08:00 h (A and B) and 18:30-20:00 h. (C and D) for Cholesterol-7a-hydroxylase; Cyp7a1 (A and C) and Sterol-27a-hydroxylase; Cyp27a1 (B and D)

In conclusion, the results presented here collectively show that voluntary wheel running in healthy chow-fed mice enhanced cholesterol turnover. The running-induced enhanced cholesterol turnover was reflected in a decreased intestinal cholesterol and bile acid absorption leading to a subsequent increase in cholesterol synthesis and alterations in bile acid metabolism. Herein, this work offers novel findings on the mechanisms contributing to the beneficial effects of exercise on cardiovascular diseases.

ACKNOWLEDGEMENTS

The authors gratefully thank Vincent W. Bloks for critical reading of the manuscript. The results of the present study do not constitute endorsement by ACSM. Authors have no professional relationships with companies or manufacturers who will benefit from the results of the present study to disclose.

REFERENCES

1 Hagg, U., Wandt, B., Bergstrom, G., Volkmann, R. and Gan, L. M., Physical exercise capacity is associated with coronary and peripheral vascular function in healthy young adults, Am J Physiol Heart Circ Physiol, 2005, 289: H1627-1634.
2 Halverstadt, A., Phares, D. A., Wilund, K. R., Goldberg, A. P. and Hagberg, J. M., Endurance exercise training raises high-density lipoprotein cholesterol and lowers small low-density lipoprotein and very low-density lipoprotein independent of body fat phenotypes in older men and women, Metabolism, 2007, 56: 444-450.
3 Durstine, J. L., Grandjean, P. W., Cox, C. A. and Thompson, P. D., Lipids, lipoproteins, and exercise, J Cardiopulm Rehabil, 2002, 22: 385-398.
4 Leaf, D. A., The effect of physical exercise on reverse cholesterol transport, Metabolism, 2003, 52: 950-957.
5 Olchawa, B., Kingwell, B. A., Hoang, A., Schneider, L., Miyazaki, O., Nestel, P. and Sviridov, D., Physical fitness and reverse cholesterol transport, Arterioscler Thromb Vasc Biol, 2004, 24: 1087-1091.
6 Glomset, J. A., The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology, Adv Lipid Res, 1973, 11: 1-65.
7 Dietschy, J. M. and Turley, S. D., Control of cholesterol turnover in the mouse, J Biol Chem, 2002, 277: 3801-3804.
8 Marschall, H. U. and Einarsson, C., Gallstone disease, J Intern Med, 2007, 261: 529-542.

9 Grundy, S. M., Ahrens, E. H., Jr. and Salen, G., Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism, J Lab Clin Med, 1971, 78: 94-121.
10 Levy, R. I., Fredrickson, D. S., Stone, N. J., Bilheimer, D. W., Brown, W. V., Glueck, C. J., Gotto, A. M., Herbert, P. N., Kwiterovich, P. O., Langer, T., LaRosa, J., Lux, S. E., Rider, A. K., Shulman, R. S. and Sloan, H. R., Cholestyramine in type II hyperlipoproteinemia. A double-blind trial, Ann Intern Med, 1973, 79: 51-58.
11 Wilund, K. R., Feeney, L. A., Tomayko, E. J., Chung, H. R. and Kim, K., Endurance exercise training reduces gallstone development in mice, J Appl Physiol, 2008, 104: 761-765.
12 Yiamouyiannis, C. A., Martin, B. J. and Watkins, J. B., 3rd, Chronic physical activity alters hepatobiliary excretory function in rats, J Pharmacol Exp Ther, 1993, 265: 321-327.
13 Watkins, J. B., 3rd, Crawford, S. T. and Sanders, R. A., Chronic voluntary exercise may alter hepatobiliary clearance of endogenous and exogenous chemicals in rats, Drug Metab Dispos, 1994, 22: 537-543.
14 Allen, D. L., Harrison, B. C., Maass, A., Bell, M. L., Byrnes, W. C. and Leinwand, L. A., Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse, J Appl Physiol, 2001, 90: 1900-1908.
15 Binder, E., Droste, S. K., Ohl, F. and Reul, J. M., Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice, Behav Brain Res, 2004, 155: 197-206.
16 Luger, A., Deuster, P. A., Kyle, S. B., Gallucci, W. T., Montgomery, L. C., Gold, P. W., Loriaux, D. L. and Chrousos, G. P., Acute hypothalamic-pituitary-adrenal responses to the stress of treadmill exercise. Physiologic adaptations to physical training, N Engl J Med, 1987, 316: 1309-1315.
17 Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 1959, 37: 911-917.
18 Miettinen, T. A., Tilvis, R. S. and Kesaniemi, Y. A., Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population, Am J Epidemiol, 1990, 131: 20-31.
19 Windler, E., Zyriax, B. C., Kuipers, F., Linseisen, J. and Boeing, H., Association of plasma phytosterol concentrations with incident coronary heart disease Data from the CORA study, a case-control study of coronary artery disease in women, Atherosclerosis, 2009, 203: 284-290.
20 Mashige, F., Imai, K. and Osuga, T., A simple and sensitive assay of total serum bile acids, Clin Chim Acta, 1976, 70: 79-86.
21 Arca, M., Montali, A., Ciocca, S., Angelico, F. and Cantafora, A., An improved gas-liquid chromatographic method for the determination of fecal neutral sterols, J Lipid Res, 1983, 24: 332-335.
22 Setchell, K. D., Lawson, A. M., Tanida, N. and Sjovall, J., General methods for the analysis of metabolic profiles of bile acids and related compounds in feces, J Lipid Res, 1983, 24: 1085-1100.
23 Plosch, T., Kok, T., Bloks, V. W., Smit, M. J., Havinga, R., Chimini, G., Groen, A. K. and Kuipers, F., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1, J Biol Chem, 2002, 277: 33870-33877.
24 Lefever, S., Vandesompele, J., Speleman, F. and Pattyn, F., RTPrimerDB: the portal for real-time PCR primers and probes, Nucleic Acids Res, 2009, 37: D942-945.
25 Kuipers, F., Havinga, R., Bosschieter, H., Toorop, G. P., Hindriks, F. R. and Vonk, R. J., Enterohepatic circulation in the rat, Gastroenterology, 1985, 88: 403-411.
26 Hulzebos, C. V., Wolters, H., Plosch, T., Kramer, W., Stengelin, S., Stellaard, F., Sauer, P. J., Verkade, H. J. and Kuipers, F., Cyclosporin a and enterohepatic circulation of

bile salts in rats: decreased cholate synthesis but increased intestinal reabsorption, J Pharmacol Exp Ther, 2003, 304: 356-363.
27 Hui, D. Y., Labonte, E. D. and Howles, P. N., Development and physiological regulation of intestinal lipid absorption. III. Intestinal transporters and cholesterol absorption, Am J Physiol Gastrointest Liver Physiol, 2008, 294: G839-843.
28 Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C. and Hobbs, H. H., Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol, J Clin Invest, 2002, 110: 671-680.
29 Graf, G. A., Li, W. P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C. and Hobbs, H. H., Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface, J Clin Invest, 2002, 110: 659-669.
30 Sutherland WH, N. E., Macfarlane DJ, Robertson MC, Williamson SA, Fecal bile acid concentration in distance runners., Int J Sports Med, 1991, 12: 533-536.
31 Sutherland WH, N. E., Macfarlane DJ, Williamson SA, Robertson MC., Cholesterol metabolism n distance runners., Clin Physiol., 1992, 12: 29-37.
32 Sutherland WH, N. E., Boulter CP, Shelling A., Physical training plasma lipoproteins and faecal steroid excretion in sedentary men., Clin Physiol., 1988, 8: 445-452.
33 Hofmann, A. F. and Hagey, L. R., Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics, Cell Mol Life Sci, 2008, 65: 2461-2483.
34 Matsumoto, M., Inoue, R., Tsukahara, T., Ushida, K., Chiji, H., Matsubara, N. and Hara, H., Voluntary running exercise alters microbiota composition and increases n-butyrate concentration in the rat cecum, Biosci Biotechnol Biochem, 2008, 72: 572-576.
35 Kidambi, S. and Patel, S. B., Cholesterol and non-cholesterol sterol transporters: ABCG5, ABCG8 and NPC1L1: a review, Xenobiotica, 2008, 38: 1119-1139.
36 van der Veen, J. N., Kruit, J. K., Havinga, R., Baller, J. F., Chimini, G., Lestavel, S., Staels, B., Groot, P. H., Groen, A. K. and Kuipers, F., Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1, J Lipid Res, 2005, 46: 526-534.
37 Wilund, K. R., Feeney, L. A., Tomayko, E. J., Weiss, E. P. and Hagberg, J. H., Effects of endurance exercise training on markers of cholesterol absorption and synthesis, Physiol Res, 2008.
38 Varady, K. A., Ebine, N., Vanstone, C. A., Parsons, W. E. and Jones, P. J., Plant sterols and endurance training combine to favorably alter plasma lipid profiles in previously sedentary hypercholesterolemic adults after 8 wk, Am J Clin Nutr, 2004, 80: 1159-1166.
39 Noshiro, M., Nishimoto, M. and Okuda, K., Rat liver cholesterol 7 alpha-hydroxylase. Pretranslational regulation for circadian rhythm, J Biol Chem, 1990, 265: 10036-10041.
40 Liu, Y., Havinga, R., FR, V. D. L., Boverhof, R., Sauer, P. J., Kuipers, F. and Stellaard, F., Dexamethasone exposure of neonatal rats modulates biliary lipid secretion and hepatic expression of genes controlling bile acid metabolism in adulthood without interfering with primary bile acid kinetics, Pediatr Res, 2008, 63: 375-381.

CHAPTER 5

Voluntary exercise increases cholesterol efflux but not macrophage reverse cholesterol transport *in vivo* in mice

M.Meissner*, N. Nijstad*, F. Kuipers, U. J.F. Tietge
* These authors contributed equally to this study
Nutr Metab 2010 Jul 1; 7:54

ABSTRACT

Physical exercise beneficially impacts on the plasma lipoprotein profile as well as on the incidence of cardiovascular events and is therefore recommended in primary and secondary prevention strategies against atherosclerotic cardiovascular disease. However, the underlying mechanisms of the protective effect of exercise remain largely unknown. Therefore, the present study tested the hypothesis that voluntary exercise in mice impacts on cholesterol efflux and *in vivo* reverse cholesterol transport (RCT). After two weeks of voluntary wheel running (average 10.1 ± 1.4 km/day) plasma triglycerides were lower ($p<0.05$), while otherwise lipid and lipoprotein levels did not change. Macrophage cholesterol efflux towards plasma was significantly increased in running (n=8) compared to sedentary (n=6) mice (14.93 ± 1.40 vs. 12.33 ± 2.60%, $p<0.05$). In addition, fecal excretion of bile acids (3.86 ± 0.50 vs. 2.90 ± 0.51 nmol/d, $p=0.001$) and neutral sterols (2.75 ± 0.43 vs. 1.94 ± 0.22 nmol/d, $p<0.01$) was significantly higher in running mice. However, RCT from macrophages to feces remained essentially unchanged in running mice compared with sedentary controls (bile acids: 3.2 ± 1.0 vs. 2.9 ± 1.1 % of injected dose, n.s.; neutral sterols: 1.4 ± 0.7 vs. 1.1 ± 0.5 % injected dose, n.s.). Judged by the plasma lathosterol to cholesterol ratio, endogenous cholesterol synthesis was increased in exercising mice (0.15 ± 0.03 vs. 0.11 ± 0.02, $p<0.05$), while the hepatic mRNA expression of key transporters for biliary cholesterol (Abcg5/g8, Sr-bI) as well as bile acid (Abcb11) and phospholipd (Abcb4) excretion did not change. These data indicate that the beneficial effects of exercise on cardiovascular health include increased cholesterol efflux, but do not extend to other components of RCT. The increased fecal cholesterol excretion observed in running mice is likely explained by higher endogenous cholesterol synthesis, however, it does not reflect increased RCT in the face of unchanged expression of key transporters for biliary sterol secretion.

INTRODUCTION

Complications of atherosclerotic cardiovascular disease (CVD) represent a major cause of morbidity and mortality in developed societies [1]. Physical exercise is associated with a reduced risk for coronary events and is therefore recommended for primary as well as secondary prevention strategies [2, 3]. As one potential beneficial effect physical exercise has been shown to improve the plasma lipoprotein profile towards a less atherogenic phenotype [4, 5]. In addition, exercise in humans increases the capacity of plasma to promote cholesterol efflux from RAW-264.7 mouse macrophages *in vitro*[6]. However, the impact of exercise on *in vivo* macrophage-to-feces reverse cholesterol transport (RCT) has not been addressed. Therefore, the present study assessed the impact of voluntary exercise in mice on cholesterol efflux and *in vivo* RCT.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice from Charles River (L'Arbresle, France) were kept in rooms with alternating 12-hour periods of light (from 7:00 a.m. to 7:00 p.m.) and dark (from 7:00 p.m. to 7:00 a.m.), with *ad libitum* access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with national laws and were approved by the responsible ethics committee of the University of Groningen.

Voluntary cage wheel running experiments

Twelve week old mice were individually housed in cages either equipped with an 11 cm steel running wheel continuously present in the cage (running group, n=8) or not (sedentary control group, n=6) [7]. Distance covered and time of running were recorded daily during the two week experimental period with a digital cycling computer (K-13-TL SET-P3-NL, Xiron, The Netherlands).

Cholesterol efflux and *in vivo* RCT

Thioglycollate-elicited mouse peritoneal macrophages were harvested and cultured essentially as described [8]. Macrophages were loaded for 24h with 50 µg/ml acetylated LDL and 3 µCi/ml ^{3}H-cholesterol (Perkin Elmer, Boston, MA, USA) and equilibrated for 18h in RPMI 1640 medium containing 1% penicillin/streptomycin and 2% bovine serum albumin (Sigma, St. Louis, MO, USA). For *in vitro* cholesterol efflux experiments, cells were incubated for 24h with 1% of respective plasma samples (performed in triplicates). Efflux was determined as the percentage of label in the supernatant related to the total amount of label within medium and cells [9]. For *in vivo* RCT two million labeled macrophages were injected intraperitoneally, blood samples were taken at 6, 24 and 48h, feces collected for 48h, and after 48h livers were harvested (sacrifice by heart puncture under isoflurane anesthesia) and stored at -80°C until further analysis as previously published [9]. Plasma counts were assessed directly by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT, USA). Counts within liver were determined following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) exactly as reported [10]. Fecal samples were dried, weighed and thoroughly ground. Aliquots were separated into bile acid and neutral sterol fractions as previously published [11]. Counts recovered from respective aliquots were related to the total amount of feces produced over 48h. All obtained counts were expressed relative to the administered tracer dose.

Plasma lipid and lipoprotein analysis

Plasma total cholesterol and triglycerides were measured enzymatically (Wako Pure Chemical Industries, Neuss, Germany). To determine plasma HDL cholesterol levels, apoB-containing lipoproteins were precipitated using 0.36% phosphotungstic acid (Sigma) and cholesterol content in the supernatant was determined as described above. Pooled plasma samples from mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described [11]. Sample separation took place at a flow of 0.5 ml/minand were collected in 500 µl fractions. Individual fractions were assayed for cholesterol

concentrations as described above. Plasma lathosterol levels relative to plasma cholesterol levels were measured by gas chromatography as described [12].

Liver lipid analysis

Liver lipids were extracted following the general procedure of Bligh and Dyer and were determined enzymatically using commercially available reagents (Wako Pure Chemical Industries, Neuss, Germany) [13].

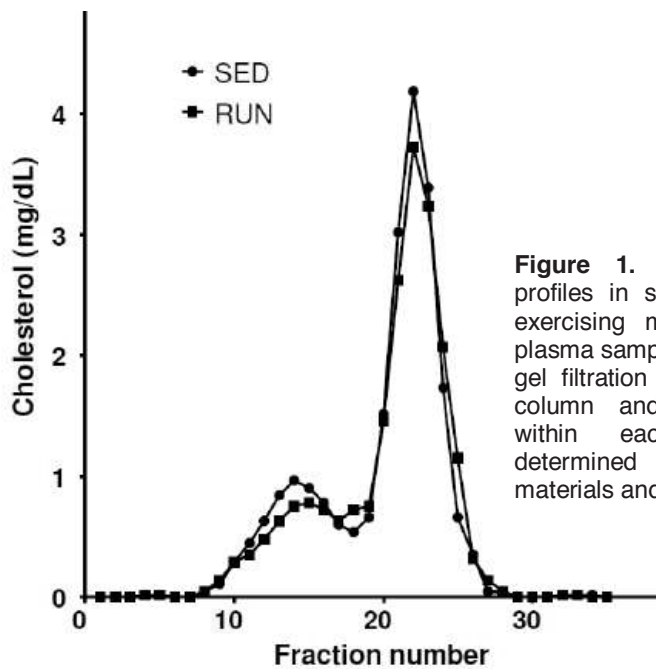


Figure 1. FPLC cholesterol profiles in sedentary (SED) and exercising mice (RUN). Pooled plasma samples were subjected to gel filtration using a superose 6 column and cholesterol levels within each fraction were determined as described in materials and methods.

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was isolated using Tri-Reagent (Sigma), and real-time quantitative PCR was carried out on an ABI-Prism 7700 (Applied Biosystems, Foster City, CA, USA) sequence detector with the default settings [14]. PCR primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems). mRNA expression levels were calculated relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA). Values are expressed as means ± SD. Student´s *t* test was used to assess statistical differences between groups. Statistical significance for all comparisons was assigned at $P<0.05$.

RESULTS

Voluntary exercise decreases hepatic cholesterol content, while plasma cholesterol levels remain unchanged

Exercising mice ran almost exclusively during the dark cycle, on average 356±52 min/d thereby covering a distance of 10.2±2.2 km (average speed: 1.78±0.18 km/h). Plasma triglycerides were lower ($p<0.05$), while phospholipids, total, non-HDL and HDL

Table 1. Plasma and liver lipids. Values are means ± SD in sedentary (SED n=6) and mice exposed to a voluntary wheel (RUN, n=7) at 2 weeks of voluntary wheel running exercise. * $p<0.05$ *vs.* sedentary mice

	SED	RUN
PLASMA LIPIDS		
Total cholesterol (mg/dl)	85 ± 3	78 ± 7
HDL cholesterol (mg/dl)	56 ± 5	49 ± 6
Non-HDL cholesterol (mg/dl)	29 ± 4	29 ± 6
Triglycerides (mg/dl)	68 ± 20	44 ± 16*
Phospholipids (mg/dl)	182 ± 7	155 ± 23
MORPHOLOGICAL DATA		
Body weight (g)	22.2 ± 1.1	21.8 ± 1.3
Liver weight (g)	0.98 ± 0.06	1.1 ± 0.06*
Liver weight (% of body weight)	4.4 ±0.2	5.0 ± 0.3*
Food intake (g/d)	4.1 ± 0.3	5.4 ± 0.5*
LIVER LIPIDS		
Total cholesterol (µmol/g)	7.3 ± 0.8	6.3 ± 0.7
Free cholesterol (µmol/g)	6.6 ± 0.8	5.6 ± 0.7*
Cholesterol esters (µmol/g)	0.7 ± 0.3	0.6 ± 0.1
Triglycerides (µmol/g)	24.3 ± 3.6	14.0 ± 2.3*
Phospholipids (µmol/g)	37.8 ± 5.5	33.3 ± 5.3

cholesterol (Table 1) and apoA-I (Western blot, data not shown) remained unchanged in response to exercise. FPLC analysis revealed a small decrease in the HDL and VLDL/LDL cholesterol peaks of running mice (Figure 1). Running increased liver weight by 12% ($p<0.01$, Table 1), while hepatic cholesterol and triglyceride contents were decreased by 14% ($p<0.05$) and 42% ($p<0.001$), respectively.

Cholesterol efflux from macrophage foam cells towards plasma of running mice is increased

Although plasma total cholesterol levels did not change and HDL cholesterol levels tended to be lower in running mice, *in vitro* cholesterol efflux towards plasma of the running mice was significantly increased ($p<0.001$, Figure 2). This effect was consistent independent of the blood sampling time from these mice, either directly after (a.m.) or before (p.m.) the running period.

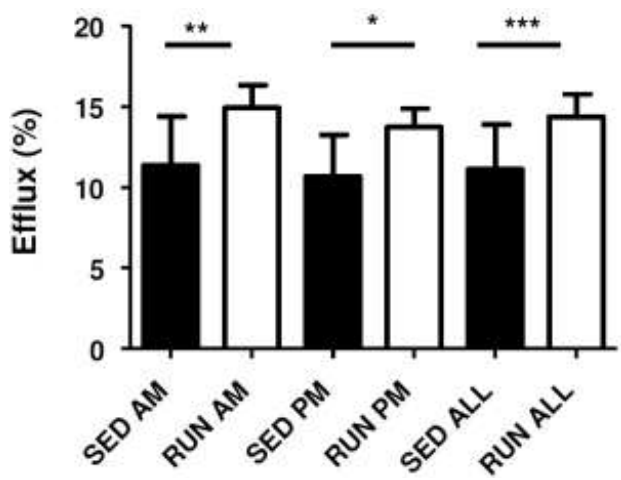


Figure 2: *In vitro* cholesterol efflux from macrophage foam cells towards plasma from sedentary (SED) and exercising mice (RUN). Efflux experiments were performed using primary mouse peritoneal macrophages and 1% plasma from the respective mice as detailed in materials and methods. Running wheels were continuously present in the cage, however, running activity occurred almost exclusively at night during the dark cycle. In order to determine, if changes in cholesterol efflux might be short-term effects occuring directly after the running period, plasma from mice bled in the morning immediately after running (AM) and bled directly before the dark cycle after rest during the day (PM) was compared separately. Data are given as means ± SD; n=6 each for SED AM and PM; n=9 for RUN PM; n=8 for RUN AM; n=12 for SED ALL and n=17 for RUN ALL. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Macrophage-to-feces RCT is unchanged in exercising mice

Next, *in vivo* RCT experiments were performed. ^{3}H-cholesterol originating from macrophages was unchanged in plasma comparing running with sedentary mice as were counts within liver (Figure 3). Daily feces production (871±97 vs. 616±54 mg/d, p<0.001) and mass fecal excretion of bile acids (3.86±0.50 vs. 2.90±0.51 nmol/d,

Table 2. Hepatic gene expression levels. mRNA expression levels were determined by real-time quantitative PCR in livers of C57BL/6 mice in sedentary (SED, n=6) and mice exposed to a voluntary running wheel (RUN, n=7) after 2 weeks of voluntary wheel running exercise compared. Results are normalized to the expression of the housekeeping gene cyclophilin and are expressed relative to the respective controls. Data are given as means ± SD.

	SED	RUN
Abcg5	1.0 ± 0.3	1.2 ± 0.3
Abcg8	1.0 ± 0.2	1.3 ± 0.3
Sr-bl	1.0 ± 0.1	1.0 ±0.2
Abcb11	1.0 ± 0.1	1.1 ± 0.2
Abcb4	1.0 ± 0.2	1.1 ± 0.2

p=0.001) and neutral sterols (2.70±0.25 vs. 1.90±0.39 nmol/d, p<0.01) were increased in the running group. However, fecal excretion of ^{3}H-cholesterol tracer originating from macrophages, reflecting completed RCT, remained unchanged both within neutral sterols and bile acids (Figure 3). In support of these physiological data, also the mRNA expression of several transporters critical for the biliary secretion of cholesterol (Abcg5/g8, Sr-bl), bile acids (Abcb11) and phospholipids (Abcb4) remained unchanged (Table 2). The plasma lathosterol/cholesterol ratio as a measure of endogenous cholesterol synthesis was significantly higher in the running mice (Figure 4) indicating that the increased amount of fecal sterols secreted in this group is rather originating from increased cholesterol synthesis than reflecting increased RCT.

DISCUSSION

The results of this study demonstrate that voluntary exercise in mice increases the efflux capacity of plasma despite a tendency towards decreased plasma HDL cholesterol levels, but does not alter macrophage-to-feces RCT. To the best of our

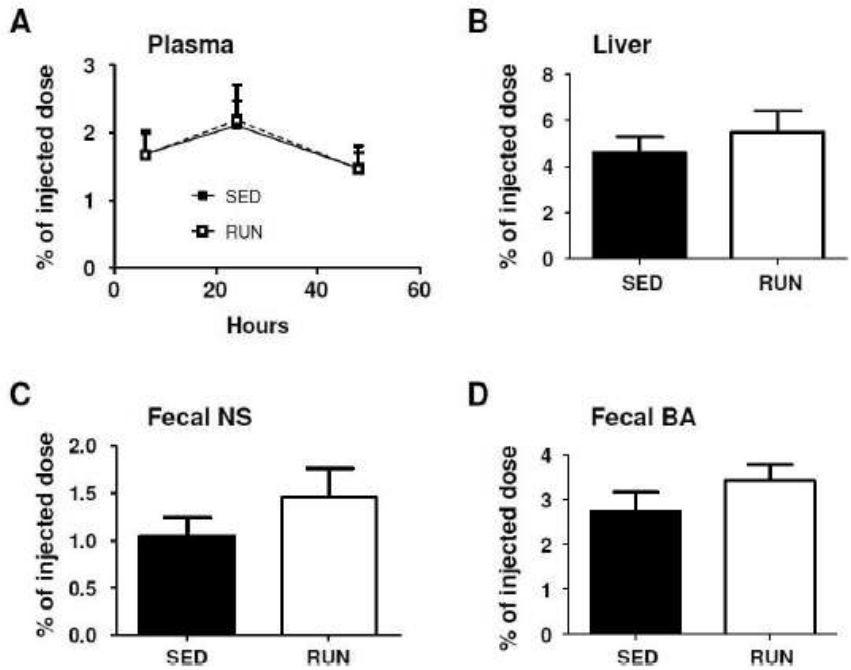


Figure 3: *In vivo* reverse cholesterol transport in sedentary (SED) and exercising (RUN) mice. At the end of the 2-week experimental period exercising mice (n=8) and sedentary controls (n=6) were injected intraperitoneally with ^{3}H-cholesterol-labeled and acLDL-loaded primary mouse peritoneal macrophages and tracer appearance was followed for 48h as detailed in materials and methods. (A) Time course of tracer appearance in plasma, (B) tracer recovery within liver at the 48h time point, (C) tracer level within fecal bile acids (BA) after 48h, (D) tracer recovery within fecal neutral sterols (NS) after 48h. Data are expressed as percentage of the injected tracer dose and are given as means ± SD.

knowledge this is the first study investigating a potential impact of exercise on *in vivo* RCT. While the beneficial effects of exercise on cardiovascular health have long been noted and exercise has been implemented in the recommendations for primary as well as secondary prevention strategies [3], the precise underlying mechanism for exercise decreasing CVD risk has not been fully elucidated thus far. It has been noted that aerobic capacity training decreases markers of inflammation and oxidative stress as well as blood pressure levels over time [3, 15, 16], while acute endurance exercise such as marathon running is associated with increased oxidative stress and a pro-inflammatory response [17, 18]. As a further beneficial effect of exercise in humans increased plasma HDL cholesterol levels and an increased capacity of plasma from exercising individuals to stimulate cholesterol

efflux from macrophage foam cells *in vitro* has been noted [6]. These results have been related to an increased plasma level of preβ-HDL particles in

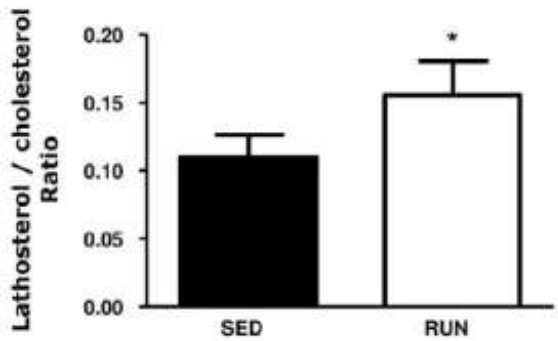


Figure 4: Effect of two week voluntary wheel running on the plasma lathosterol / cholesterol ratio as marker of endogenous cholesterol synthesis in exercising mice (RUN, n=8) and sedentary controls (SED, n=6). Plasma cholesterol and lathosterol levels were determined by gas chromatography as detailed in materials and methods. Data are given as means ± SD

trained individuals [19, 20]. Our study confirmed the efflux data in a mouse model of voluntary endurance exercise, while in contrast to the human situation plasma HDL cholesterol levels rather had a tendency to be lower in exercising mice. The increase in cholesterol efflux *in vitro* was not reflected by altered plasma counts in the *in vivo* RCT experiment, likely since these represent the net balance between efflux and removal from the plasma compartment. Interestingly, feces production was increased in exercising mice, which also mirrors the human situation [21]. In addition, fecal mass secretion of bile acids and neutral sterols was significantly elevated in response to exercise, which in our interpretation reflects increased endogenous hepatic cholesterol synthesis. In humans one study reported a non-significant 63% increase in endogenous cholesterol synthesis using the deuterium incorporation method [22], while another study detected no difference in plasma lathosterol levels in response to exercise training [23]. Since RCT in humans differs in several aspects from mice (e.g. by the expression of CETP), studies on the impact of exercise on RCT in humans will be interesting to perform, once an integrated experimental system for these types of studies becomes available.

REFERENCES

1 Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., De Simone, G., Ferguson, T. B., Ford, E., Furie, K., Gillespie, C., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, P. M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M. M., Meigs, J., Mozaffarian, D., Mussolino, M., Nichol, G., Roger, V. L., Rosamond, W., Sacco, R., Sorlie, P., Thom, T., Wasserthiel-Smoller, S., Wong, N. D. and Wylie-Rosett, J., Heart disease and stroke statistics--2010 update: a report from the American Heart Association, Circulation, 121: e46-e215.

2 Deedwania, P., Kosiborod, M., Barrett, E., Ceriello, A., Isley, W., Mazzone, T. and Raskin, P., Hyperglycemia and acute coronary syndrome: a scientific statement from the American Heart Association Diabetes Committee of the Council on Nutrition, Physical Activity, and Metabolism, Anesthesiology, 2008, 109: 14-24.

3 Thompson, P. D., Buchner, D., Pina, I. L., Balady, G. J., Williams, M. A., Marcus, B. H., Berra, K., Blair, S. N., Costa, F., Franklin, B., Fletcher, G. F., Gordon, N. F., Pate, R. R., Rodriguez, B. L., Yancey, A. K. and Wenger, N. K., Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease: a statement from the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity), Circulation, 2003, 107: 3109-3116.

4 Leon, A. S. and Sanchez, O. A., Response of blood lipids to exercise training alone or combined with dietary intervention, Med Sci Sports Exerc, 2001, 33: S502-515; discussion S528-509.

5 Leon, A. S., Rice, T., Mandel, S., Despres, J. P., Bergeron, J., Gagnon, J., Rao, D. C., Skinner, J. S., Wilmore, J. H. and Bouchard, C., Blood lipid response to 20 weeks of supervised exercise in a large biracial population: the HERITAGE Family Study, Metabolism, 2000, 49: 513-520.

6 Olchawa, B., Kingwell, B. A., Hoang, A., Schneider, L., Miyazaki, O., Nestel, P. and Sviridov, D., Physical fitness and reverse cholesterol transport, Arterioscler Thromb Vasc Biol, 2004, 24: 1087-1091.

7 Allen, D. L., Harrison, B. C., Maass, A., Bell, M. L., Byrnes, W. C. and Leinwand, L. A., Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse, J Appl Physiol, 2001, 90: 1900-1908.

8 Tietge, U. J., Pratico, D., Ding, T., Funk, C. D., Hildebrand, R. B., Van Berkel, T. and Van Eck, M., Macrophage-specific expression of group IIA sPLA2 results in accelerated atherogenesis by increasing oxidative stress, J Lipid Res, 2005, 46: 1604-1614.

9 Annema, W., Nijstad, N., Tolle, M., de Boer, J. F., Buijs, R. V., Heeringa, P., van der Giet, M. and Tietge, U. J., Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A(2), J Lipid Res, 51: 743-754.

10 Nijstad, N., Wiersma, H., Gautier, T., van der Giet, M., Maugeais, C. and Tietge, U. J., Scavenger receptor BI-mediated selective uptake is required for the remodeling of high density lipoprotein by endothelial lipase, J Biol Chem, 2009, 284: 6093-6100.

11 Wiersma, H., Gatti, A., Nijstad, N., Kuipers, F. and Tietge, U. J., Hepatic SR-BI, not endothelial lipase, expression determines biliary cholesterol secretion in mice, J Lipid Res, 2009, 50: 1571-1580.

12 Windler, E., Zyriax, B. C., Kuipers, F., Linseisen, J. and Boeing, H., Association of plasma phytosterol concentrations with incident coronary heart disease Data from the CORA study, a case-control study of coronary artery disease in women, Atherosclerosis, 2009, 203: 284-290.

13 Wiersma, H., Gatti, A., Nijstad, N., Oude Elferink, R. P., Kuipers, F. and Tietge, U. J., Scavenger receptor class B type I mediates biliary cholesterol secretion independent of ATP-binding cassette transporter g5/g8 in mice, Hepatology, 2009, 50: 1263-1272.

14 Wiersma, H., Nijstad, N., Gautier, T., Iqbal, J., Kuipers, F., Hussain, M. M. and Tietge, U. J., Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice, J Lipid Res, 51: 544-553.
15 Agarwal, D., Haque, M., Sriramula, S., Mariappan, N., Pariaut, R. and Francis, J., Role of proinflammatory cytokines and redox homeostasis in exercise-induced delayed progression of hypertension in spontaneously hypertensive rats, Hypertension, 2009, 54: 1393-1400.
16 Cornelissen, V. A. and Fagard, R. H., Effects of endurance training on blood pressure, blood pressure-regulating mechanisms, and cardiovascular risk factors, Hypertension, 2005, 46: 667-675.
17 Kratz, A., Wood, M. J., Siegel, A. J., Hiers, J. R. and Van Cott, E. M., Effects of marathon running on platelet activation markers : direct evidence for in vivo platelet activation, Am J Clin Pathol, 2006, 125: 296-300.
18 Gomez-Cabrera, M. C., Martinez, A., Santangelo, G., Pallardo, F. V., Sastre, J. and Vina, J., Oxidative stress in marathon runners: interest of antioxidant supplementation, Br J Nutr, 2006, 96 Suppl 1: S31-33.
19 Jafari, M., Leaf, D. A., Macrae, H., Kasem, J., O'Conner, P., Pullinger, C., Malloy, M. and Kane, J. P., The effects of physical exercise on plasma prebeta-1 high-density lipoprotein, Metabolism, 2003, 52: 437-442.
20 Sviridov, D., Kingwell, B., Hoang, A., Dart, A. and Nestel, P., Single session exercise stimulates formation of pre beta 1-HDL in leg muscle, J Lipid Res, 2003, 44: 522-526.
21 Sutherland, W. H., Nye, E. R., Macfarlane, D. J., Robertson, M. C. and Williamson, S. A., Fecal bile acid concentration in distance runners, Int J Sports Med, 1991, 12: 533-536.
22 Varady, K. A., Houweling, A. H. and Jones, P. J., Effect of plant sterols and exercise training on cholesterol absorption and synthesis in previously sedentary hypercholesterolemic subjects, Transl Res, 2007, 149: 22-30.
23 Wilund, K. R., Feeney, L. A., Tomayko, E. J., Weiss, E. P. and Hagberg, J. M., Effects of endurance exercise training on markers of cholesterol absorption and synthesis, Physiol Res, 2009, 58: 545-552.

CHAPTER 6

Voluntary wheel running decreases atherosclerosis development and increases sterol excretion in hypercholesterolemic mice

M. Meissner, E. Lombardo, R. Havinga, U.J.F. Tietge, F. Kuipers, A. K. Groen

Submitted

ABSTRACT

Objective: Regular physical activity decreases the risk for atherosclerosis but underlying mechanisms are not fully understood. We questioned whether voluntary wheel running provokes specific modulations in cholesterol turnover that translate into a decreased atherosclerotic burden in hypercholesterolemic mice.

Methods: Male LDLR-deficient mice (8 wk old) had either access to a voluntary running wheel for 12 weeks (RUN) or remained sedentary (CONTROL). Both groups were fed a western-type/high cholesterol diet. Running activity and food intake were recorded. At 12 weeks of intervention, feces, bile and plasma were collected to determine fecal, biliary and plasma parameters of cholesterol metabolism and plasma cytokines. Atherosclerotic lesion size was determined in the aortic root.

Results: RUN weighed less (~13%) while food consumption was increased by 17% (p=0.004). Plasma cholesterol levels were decreased by 12% (p=0.035) and plasma levels of pro-atherogenic lipoproteins decreased in RUN compared to CONTROL. Running modulated cholesterol catabolism by enhancing cholesterol turnover: RUN displayed an increased biliary bile acid secretion (68%, p=0.007) and increased fecal bile acid (93%, p=0.009) and neutral sterols (33%, p=0.002) outputs compared to CONTROL indicating that reverse cholesterol transport was increased in RUN. Importantly, aortic lesion size was decreased by 43.6% in RUN (p=0.033).

Conclusion: Voluntary wheel running reduces atherosclerotic burden in hypercholesterolemic mice. An increased cholesterol turnover, specifically its conversion into bile acids, may underlie the beneficial effect of voluntary exercise in mice.

INTRODUCTION

Atherosclerosis is a complex vascular disease, which is characterized by major abnormalities in systemic factors, such as circulating lipids and lipoproteins, and concomitant inflammation of the vascular wall.

It has long been known that exercise is a deterrent of atherosclerosis. Numerous clinical and experimental studies report on the beneficial effects of physical activity on atherosclerosis[1-7] and various effects of physical activity on different processes involved in the pathogenesis and progression of atherosclerosis have been reported. For example, it has been shown that physical activity improves the antioxidant system[4, 6], plaque composition as well as plaque stability[3, 7] and favorably modulates the inflammatory response [1]. However, despite these recent efforts it remains unclear how exactly physical activity decreases the atherosclerotic process. We hypothesize that the enterohepatic system, which plays a critical role in several aspects of cholesterol metabolism, may be of great relevance herein.

Increasing cholesterol excretion into feces as neutral sterols or bile acids represents an efficient strategy in the amelioration of atherosclerosis, as it improves the pro-atherogenic state by modulating lipid content in plasma [8, 9]. The liver secretes free cholesterol into bile, which is released into the intestine upon ingestion of a meal. In the small intestine, biliary cholesterol mixes with dietary cholesterol and is partially reabsorbed. The remainder is lost in the feces within the neutral sterol fraction. Bile acids are synthesized from cholesterol exclusively in the liver and enter the intestinal lumen after a meal. Bile acids are important for the emulsification and absorption of dietary fats in the intestine [10]. About 95% of the bile acids are reabsorbed from the terminal ileum, transported back to the liver for re-secretion into bile (enterohepatic circulation). The fraction of bile acids that escapes reabsorption is lost in feces and constitutes an important part of cholesterol turnover, since fecal bile acid loss is compensated for by *de novo* synthesis from cholesterol to maintain the bile acid pool size[11]. Under steady state conditions, fecal bile acid loss equals hepatic *de novo* bile acid synthesis.

We have recently shown that exposing healthy chow-fed mice to a voluntary running wheel for two weeks enhanced fecal neutral sterol and bile acid excretion with specific changes in biliary, plasma and intestinal parameters contributing to an increased cholesterol turnover upon running [12]. To our knowledge, no previous studies have examined the effects of exercise on cholesterol and bile acid metabolism in a hypercholesterolemic mouse model. Thus, the purpose of this study was to investigate whether the recently observed effects of voluntary running wheel exercise on whole body cholesterol turnover in healthy chow-fed mice [12] extend to the hypercholesterolemic LDLR-deficient mouse model. We hypothesized that voluntary wheel running beneficially modulates cholesterol and bile acid metabolism in hypercholesterolemic mice and thereby mediates a reduction in atherosclerotic burden.

MATERIALS AND METHODS

All experiments were approved by the Animal Care and Use Committee of the University of Groningen, The Netherlands. The University of Groningen is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

Animals and voluntary cage-wheel exercise

Sixteen 5-week-old male LDLR deficient (B6.129S7-*LDLR*^{tm1Her}/J**)** mice were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME, USA). Upon arrival, mice were singly housed in a cage (47 x 26 x 14.5 cm) in a temperature-controlled room with a 12:12h light-dark cycle and had access to standard commercial pelleted laboratory chow (RMH-B, ABDiets, Woerden, The Netherlands). At 8 weeks of age, mice were switched to a western-type

diet (0.25% cholesterol, 16% fat, Purified Western Diet, 4021.06, ABDiets, Woerden, The Netherlands) and were randomly selected to either voluntary cage wheel running (RUN, n=10) or to remain sedentary (CONTROL, n=7) for 12 weeks. Throughout the study, mice had *ad libitum* access to food and water. The voluntary running wheel set-up has been described previously[12]. Twice a week, mice were weighed and food intake was recorded. Two mice in the running group were excluded from all analyses because they showed no activity on the running wheel.

Experimental procedures

Fecal, plasma, biliary, hepatic and intestinal parameters were collected at the endpoint of the experiment after 12 weeks of RUN or CONTROL, i.e., at 20 weeks of age.

Fecal Parameters

Forty-eight hour feces were collected before and at 12 weeks running wheel exposure. Feces were dried, weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total bile acids by an enzymatic fluorimetric assay [13]. Neutral sterols were determined according to Setchell et al. [14].

Determination of biliary parameters of cholesterol and bile acid metabolism

After 12 weeks of CONTROL or RUN, all mice underwent gallbladder cannulation for continuous collection of bile [15]. Briefly, mice were anaesthetized by intraperitoneal injection with Hypnorm ® (1 ml·kg-1) and diazepam (10mg·kg-1). During the 30min bile collection period, mice were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1g·ml-1 for bile. Bile was stored at -20°C until analysis. Total biliary bile acids were determined by an enzymatic fluorimetric assay[16]. Biliary cholesterol and phospholipids levels were measured as described by Kuipers [17].

Determination of plasma markers of cholesterol metabolism

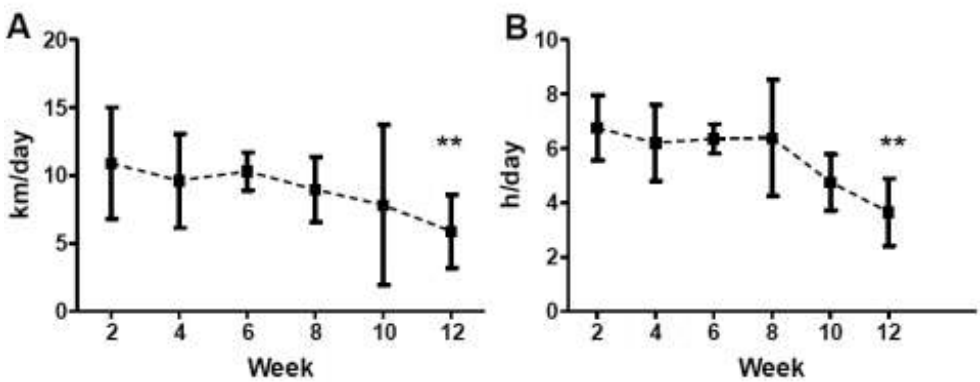
Immediately after bile collection, blood was drawn *via* the orbital sinus. Plasma was collected by centrifugation and stored at -20°C until analyzed. Plasma total cholesterol, free cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially available assay kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma pro-and anti-inflammatory makers were analyzed using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA)**.** Utilizing gaschromatography, as described by Windler et al.[18], we analyzed plasma plant sterols (campesterol and sitosterol) relative to plasma cholesterol levels as marker of intestinal cholesterol absorption in pooled plasma samples of each group. Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium).

Tissue Collection

Mice were opened immediately after blood collection. the heart was slowly perfused with PBS at physiological pressure. Then, the liver was excised, weighed and snap frozen in liquid nitrogen. The small intestine was excised, flushed with ice cold PBS (4°C) and divided into three sections of equal lengths and subsequently snap-frozen in liquid nitrogen. Lastly, the thoracic aorta was excised and epididymidal fat pads were removed and weighed. Thoracic aorta, liver and intestine were stored at -80ºC for later analysis. Hearts were flushed with PBS to remove the excess of blood before fixation in formaldehyde 1% (Formal-Fixx, Thermo Electron Corporation, Pittsburgh, Pa.) for 24 hours, cut in an angle eventually revealing the aortic sinus and stored at –80°C embedded in OCT (Tissue-Tek O.C.T., Sakura, Zoeterwoude, the Netherlands).

Determination of atherosclerotic lesion size and aortic cholesterol content Frozen sections from the aortic sinus were prepared according to Paigen *et al* [19]. Surface lesion area was measured after Oil Red O staining by

computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 video camera. At least 5 sections per mouse were examined for each staining. Due to technical difficulties, we were able to analyze atherosclerotic lesion size in 4 of 8 running mice and 4 of 7 sedentary mice.



Supplemental Figure 1. Voluntary running wheel activity during the 12 week running wheel exposure: A. Average distance ran per day (km/h) and B: Average time ran per day (h/day) in RUN (n=7). Data was statistically analyzed comparing 2 and 12 weeks utilizing the Wilcoxon signes-rank test. Data are expressed as means ± SD; *p<0.05 2 weeks vs. 12 weeks

Determination of hepatic lipids

Hepatic lipids were determined after extraction according to Bligh and Dyer [20] and redisolving in Triton-2%H_2O using the same kits as for plasma lipids. Phospholipids were determined in all tissues according to Böttcher et al [21].

RNA isolation and PCR procedures

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described by Plösch *et.al.* [15]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to β-actin.

Statistics

Statistical analysis was performed using the Mann-Whitney-U test (SPSS 12.0.1 for Windows). All data are expressed as means ± SD. *P*-values of <0.05 were considered statistically significant.

RESULTS

Running wheel activity and morphometric parameters

Mice exposed to a voluntary running wheel progressively ran less during the 12 week running wheel intervention. While a daily average running distance of ~10 km and average running duration of ~6.5 hours was observed at the start of the experiment, it dropped to ~5.5 km/day and ~4.0 hours at the end of the experiment (Supplemental Figure 1). Despite a 17% increase in food intake compared to control mice, running mice displayed a ~13% lower bodyweight and ~73% lower epididymidal white adipose tissue weight at 12 weeks of running (Table 1). Liver weight, body weight / liver weight ratio and small intestinal length were not different between running and control mice (Table 1).

Table 1. Biometrical data. Values represent mean ± SD at 12 weeks of running in CONTROL (n=7) and RUN (n=7); *P<0.05 vs. CONTROL

	CONTROL	RUN
Body weight (g)	29.6 ± 2.3	25.9 ± 1.0*
Liver weight (g)	1.35 ± 0.14	1.23 ± 0.05
Liver weight / body weight (%)	4.6 ± 0.7	4.7 ± 0.2
Small intestine length (cm)	33.3 ± 1.8	31.5 ± 1.8
Epididymal white adipose weight (g)	0.85 ± 0.25	0.23 ± 0.1*
Food Intake (g/day)	3.4 ± 0.4	4.0 ± 0.14*

Effect of voluntary wheel running on atherosclerotic lesion size and inflammatory markers

First, we investigated whether 12 weeks of voluntary wheel running beneficially affected lesion size area in atherosclerosis-prone LDLR-deficient mice. Indeed,

quantification of atherosclerotic lesion areas in aortic sinus showed a 43.6% reduction in running mice (Figure 1A-C). Because inflammation plays an important role in the pathogenesis of atherosclerosis, we also assessed the effect of running on plasma levels of inflammatory markers. Interestingly, voluntary wheel running had no effect on plasma levels of inflammatory markers (Supplemental Figure 2).

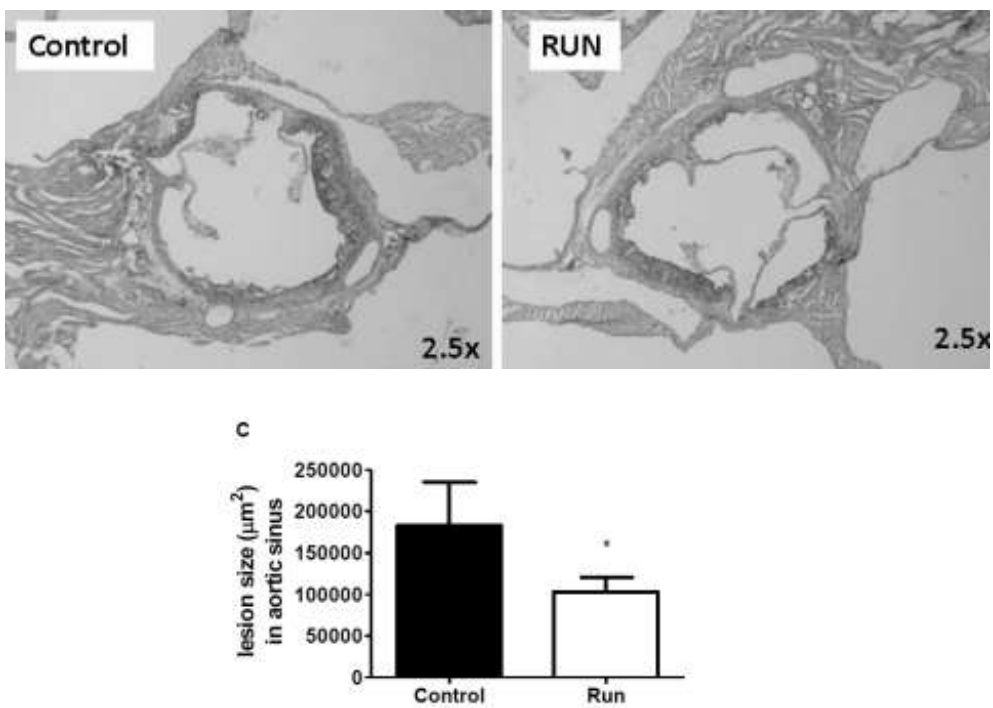
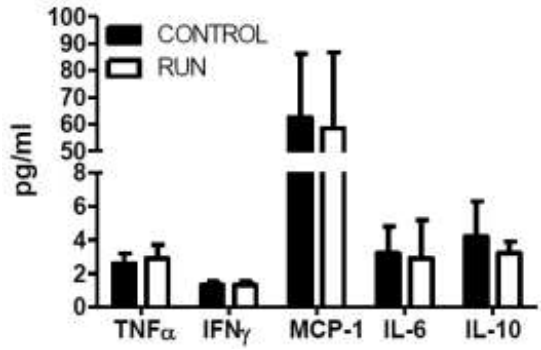


Figure 1. Voluntary wheel running reduces atherosclerotic lesions size development. Representative morphological section of aortic arch stained with Oil Red Oil of CONTROL (A) and RUN (B); (C) Quantification of lesion size in aortic sinus of Control (n=4) and RUN (n=4) at 12 weeks of running. *p<0.05 vs. CONTROL

Voluntary wheel running beneficially affects plasma lipoprotein profile

Elevated lipid levels are key factors in the development of atherosclerosis. Running mice displayed a small but significant reduction in plasma levels of total cholesterol, esterified cholesterol and triglycerides (Table 2). Importantly, we found improved plasma lipoprotein profiles with reduced levels of VLDL-and



Supplemental Figure 2. Voluntary wheel running has no effects on pro- and anti-inflammatory cytokines. Plasma cytokines in CONTROL(n=7) and RUN (n=7). Pro-inflammatory cytokines: Tumor necrosis factor alpha (TNFa), interferon gamma (IFNy), monocyte chemotactic protein 1 (MCP1), interleukin 6 (IL-6) and anti-inflammatory cytokine interleukin 10 (IL-10). Data represent means ± SD

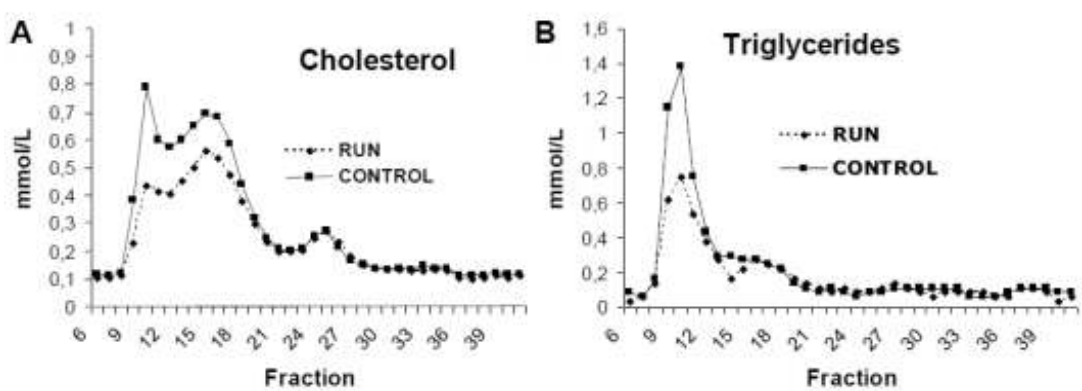
LDL-sized lipoproteins in running compared to control mice (Supplemental Figure 3A and B). Paralleling these running-induced improvements in lipoprotein profiles, we not only found a reduction in hepatic expression of microsomal triglyceride transfer protein, indicative of a decreased hepatic production of VLDL, but also observed an increased hepatic lipoprotein lipase expression (Table 3), indicative of an increased lipoprotein clearance. More beneficial effects of running were observed on hepatic lipid storage. First, control mice displayed substantial hepatic triglyceride stores, which were reduced by almost half in running mice (Table 2). Second, running resulted in a significantly lower hepatic storage of cholesterol, paralleled by a running-induced decrease in Hmgcr, the rate-limiting enzyme in cholesterol biosynthesis. Hepatic contents of cholesterol esters tended to be reduced in running mice with a lower expression of Acat2, an enzyme required for cholesterol esterification (Table 3). Moreover, the beneficial changes in hepatic lipid content were paralleled by decreased expression levels of key lipogenic genes Fasn and Scd1 (Table 3). Collectively, these data show that running provokes favorable changes in plasma and liver lipid metabolism.

Voluntary wheel running increased fecal sterol output

Next, we assessed whether the running-induced beneficial changes in plasma and liver lipid metabolism were accompanied by changes in cholesterol and bile

Table 2. Plasma and liver lipids. Values represent mean ± SD at 12 weeks of running in CONTROL (n=7) and RUN (n=7); *p<0.05 vs. CONTROL

	CONTROL	RUN
Plasma Lipids (mmol/L)		
Total Cholesterol	27.7 ± 1.5	23.6± 2.5*
Free Cholesterol	8.80 ± 1.4	7.65 ± 0.7
Cholesterol Esters	18.9 ± 0.9	15.9 ± 1.8*
Triglycerides	8.52 ± 1.54	6.59 ± 0.8*
Liver Lipids (nmol/mg liver)		
Triglycerides	78 ± 2	41 ± 10*
Total Cholesterol	16.3 ± 2.0	13.1 ± 1.3*
Free Cholesterol	5.5 ± 0.9	4.7 ± 0.3
Cholesterol Esters	10.9 ± 1.7	8.4 ± 1.1
Phospholipids	26.7 ± 2.9	23.8 ± 4.5



Supplemental Figure 3. Voluntary wheel running improves the lipoprotein profile**.** Plasma lipoprotein profiles showing distribution of cholesterol (A) and triglycerides (B) in pooled plasma of CONTROL (n=7) and RUN (n=8). Lipoproteins in plasma pools were separated by fast protein liquid chromatography (FPLC) on a Superose 6 column

acid metabolism. First, we assessed fecal parameters of cholesterol and bile acid metabolism and feces were collected quantitatively from all mice during the last 48 hours of the experiment. Hypercholesterolemic running mice had

significantly increased feces production (+19%, data not shown) as well as fecal neutral sterol (+33%) and fecal bile acid output rates (+93%) compared to sedentary controls (Table 4). No differences in any of these parameters were observed before the running wheel intervention (data not shown). Next, we investigated whether voluntary wheel running increased fecal neutral sterol output by modulating cholesterol absorption. Running had no effect on the plasma plant sterol/cholesterol ratio (Supplemental Figure 4A), a marker of cholesterol absorption. No effect of voluntary wheel running was found on jejunal Npc1l1 mRNA expression (Supplemental Figure 4B), while, intriguingly, the expression of the ATP-cassette binding transporters g5 and g8 (Supplemental Figure 4C and D), which are known to promote efflux of cholesterol and plant sterols from the enterocyte back into the intestinal lumen for elimination into feces, was increased.

Voluntary wheel running increases bile flow and biliary bile acid secretion

To evaluate whether physical activity modulates biliary parameters under hypercholesterolemia, mice were subjected to gallbladder canulations for collection of hepatic bile at 12 weeks of running. Indeed, running mice had a 22.6% increased bile

flow (P=0.011), a 67.5% increase in biliary bile acid secretion and a trend towards increased biliary cholesterol secretion (P=0.179), while no differences in the rate of biliary phospholipid secretion was found (Table 4). Moreover, no differences in the expression levels of important genes involved in cholesterol efflux from the hepatocyte towards the plasma (Table 3) were observed. Our data suggest that voluntary wheel running increases cholesterol turnover to promote its fecal excretion as cholesterol and bile acids, indicating increased cholesterol excretion out of the body in the absence of changes in endogenous cholesterol synthesis.

DISCUSSION

In the present study, we tested the hypothesis that voluntary wheel running ameliorates atherosclerosis possibly by modulating cholesterol metabolism. By using hypercholesterolemic LDLR-deficient mice on a western-type diet, we were able to show for the first time that voluntary wheel running provokes specific changes in cholesterol metabolism, particularly by promoting its conversion into bile acids likely contributing to reduced plasma lipid levels, and that these alterations coincide with a reduction in atherosclerosis.

First, we show running-induced increases in fecal neutral sterol and bile acid excretion. The fecal bile acid loss in running mice is massive and reflective of an increased *de novo* bile acid synthesis. Second, we found that the increased fecal bile acid loss was paralleled by specific changes in biliary parameters consistent with an increased cholesterol turnover. Specifically, running mice had a higher bile flow, an increased biliary bile acid secretion and a trend towards an increased biliary cholesterol secretion. Third, hepatic cholesterol content was reduced in running mice, indicating cholesterol turnover over storage.

To the best of our knowledge, this is the first study showing that voluntary exercise is equally efficient as forced exercise in reducing atherosclerotic lesion size development in hypercholesterolemic mice. While we show here a 43.6% reduction in atherosclerotic lesion size upon voluntary wheel running, previous studies in LDLR-deficient mice undergoing forced exercise, like treadmill running or swimming, reported a reduction by ~40% [4, 22]. Additionally, reductions of atherosclerotic lesion size of ~54% to ~30% have been observed in another hypercholesterolemic mouse model, the ApoE-deficient mouse, when forced to swim for different durations [5, 6, 23]. In contrast, voluntary wheel running did not result in reductions of atherosclerosis in ApoE-deficient mice with preexisting lesions, however, a reduction in pro-inflammatory markers was reported [1]. No literature is available describing the effects of exercise (forced or voluntary) on plasma pro-inflammatory markers in

Table 3. Hepatic genes involved in lipid metabolism. Hepatic mRNA expression levels of lipoprotein lipase (Lpl), microsomal triglyceride transfer protein (Mttp), 3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr), acetyltransferase 2 (Acat2), ATP-binding cassette transporter 1 (Abca1), ATP-binding cassette transporter g5 (Abcg5), ATP-binding cassette transporter 8 (Abcg8), fatty acid synthase (Fasn), stearoyl-CoA desaturase-1 (Scd1) , Values are relative to ß-actin and represent mean ± SD in Control (n=6) and RUN (n=6)

	CONTROL	RUN	*P*-value
Cholesterol Metabolism			
Clearance			
Lpl	1.0 ± 0.2	1.6 ± 0.1*	0.006
Production			
Mttp	1.0 ± 0.3	0.7 ± 0.2*	0.029
Hmgcr	1.0 ± 0.3	0.6 ± 0.1*	0.020
Acat2	1.0 ± 0.2	0.7 ± 0.1*	0.024
Efflux			
Abca1	1.0 ± 0.3	1.1 ± 0.3	0.061
Abcg5	1.0 ± 0.2	0.9 ± 0.2	0.426
Abcg8	1.0 ± 0.1	1.0 ± 0.1	0.420
Fat Metabolism			
Fasn	1.0 ± 0.2	0.7 ± 0.1*	0.048
Scd1	1.0 ± 0.3	0.6 ± 0.1*	0.029

atherosclerosis development in mice. We show here that voluntary wheel running reduced atherosclerosis independent of pro-and anti-inflammatory markers. The mechanisms behind the beneficial effects of exercise on atherosclerosis are not yet understood. Being a major physiological process for the body to clear excess cholesterol, the fecal excretion of cholesterol as neutral sterol or bile acid plays a critical role in the maintenance of whole-body cholesterol homeostasis. Increasing cholesterol excretion into feces as neutral sterol or bile acid is long known as an efficient strategy in the amelioration of atherosclerosis, as it improves the pro-atherogenic state by reducing lipid content in plasma [8, 9]**.** Furthermore, it has been demonstrated that patients with coronary artery disease have a reduced fecal excretion of bile acids [24]. Strikingly, there is a complete lack of studies describing the effects of physical activity on the enterohepatic metabolism of cholesterol in hypercholesterolemic

Table 4. Fecal and biliary sterol parameters. Values represent mean ± SD at 12 weeks of running for CONTROL (n=7) and RUN (n=7); *p<0.05 vs. CONTROL

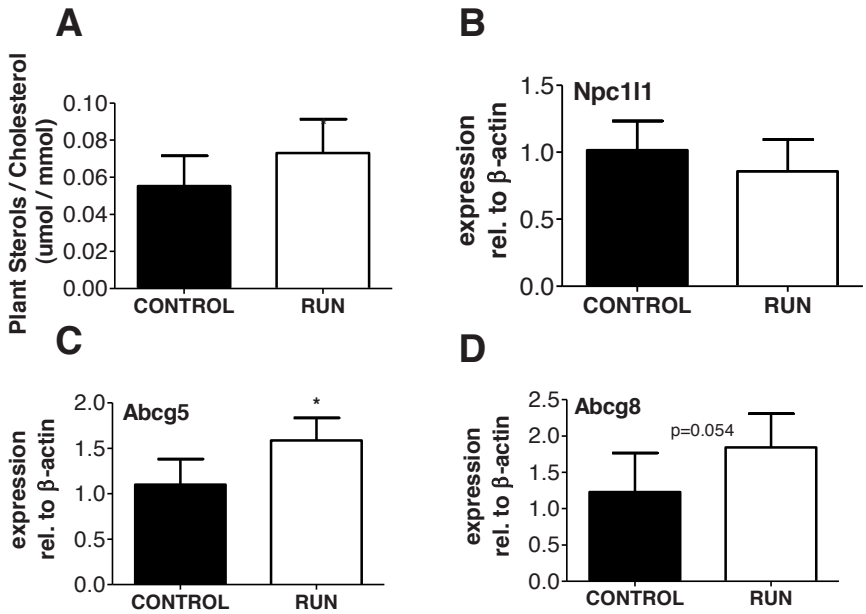
	CONTROL	RUN
Fecal outputs (µmol/24h/100gBW)		
Neutral sterols	48 ± 6	64 ± 4*
Bile acids	5.3 ± 1.6	10.2 ± 3.1*
Dietary cholesterol balance	35 ± 6	41 ± 3
Biliary secretions (µmol/24h/100gBW)		
Cholesterol	0.9 ± 0.4	1.6 ± 0.8
Bile acids	249 ± 49	417 ± 134*
Phospholipids	25.4 ± 2.9	29.4 ± 12.6
Bile flow (µl/min/100gBW)	4.3 ± 0,7	5.2 ± 0.7*

mice. Our observations of running-induced increases in fecal neutral sterol and bile acid outputs confirm our earlier results in chow-fed mice running for 2 weeks. It is, however, noteworthy that the effects are more striking in hypercholesterolemic mice running for 12 weeks. For example, while we found a ~30% increase in both fecal neutral sterol and bile acid outputs in chow-fed mice upon 2 weeks running, we report here a similar increase in fecal neutral sterol loss in 12 week running hypercholesterolemic mice (~33%) yet because the diet already contained 0,25% the amount of extra cholesterol excreted by running is massive. In addition there was a strong increase in fecal bile acid secretion (~93%) compared to 30% in control mice. This increase in fecal bile acid excretion in running mice is remarkable and reflects an increase in *de novo* bile acid synthesis. Consistent with our previous study [12] in chow-fed mice, we did not observe changes in any of the major genes involved in bile acid synthesis in running mice (*data not shown*) [12]. Thus, within this and our previous study, the increase in bile acid synthesis upon voluntary wheel running as demonstrated by fecal bile acid loss did not result in an upregulation of key genes involved in bile acid synthesis, such as Cyp7a1 and Cyp8b1 indicating that regulation via the nuclear receptor FXR and its downstream target FGF15 is not operational here. Increases in *de novo* bile acid synthesis without concomitant increases in any of the major bile acid genes have also been reported by others[25, 26] and are most likely induced by posttranscriptional mechanisms. It is speculative, therefore, that physical

activity enhances bile acid synthesis by metabolic mechanisms. For example, physical activity increased dietary fatty acid absorption (*data not shown*). During physical activity, energy is depleted in energy expending tissues, such as skeletal muscle. Thus, a high demand to recover this expended energy manifests in these tissues. In this regard, physical activity might, hypothetically, increase bile acid synthesis to increase the capacity for micelle formation, thereby fatty acid absorption and thus energy delivery to energy expending tissues.

Paralleling our observations of fecal bile acid excretion, running appears to induce more drastic effects on biliary parameters in hypercholesterolemic mice than in chow-fed healthy mice. Compared to control mice, the increase in biliary bile acid secretion was ~20% in healthy chow-fed mice running for 2 weeks [12]. Furthermore, we report here an increase in bile flow, which was not observed in running chow-fed mice, but has been previously reported in exercising rats [27].

Next, it is intriguing that the livers of running mice display ~20% less cholesterol stores and ~45% less triglyceride stores than control mice do. Control LDLR-deficient mice on a western-type diet display hepatic triglyceride and cholesterol contents more than 2.5 times that of chow-fed wildtype mice [12]. Yet, despite their increased food intake, running mice had significantly reduced hepatic cholesterol and triglyceride stores demonstrating an enhanced turnover rather than their storage. No data are available describing the effects of exercise, either forced or voluntary, on hepatic lipids in hypercholesterolemic mouse models. Yet, limited data show that high fat and low fat-fed wildtype mice displayed reduced hepatic triglycerides levels after treadmill exercise training [28] and that swim training reduced hepatic fatty acid synthesis in C57BL/6J mice [29]. Moreover, we previously found reduced hepatic triglyceride content and a trend towards reduced hepatic cholesterol content in chow-fed mice running for 2 weeks [12]. Collectively these observations demonstrate favorable effects of physical activity on hepatic lipid storage.



Supplemental Figure 4. Parameters of cholesterol absorption after 12 weeks of running. A. Plasma total plant sterol / plasma cholesterol ratio in CONTROL (n=5) and RUN (n=5); Jejunal mRNA expression levels of: B. Niemann-Pick C1 Like 1, Npc1l1; C. ATP-binding cassette transporter g5, Abcg5; and D. ATP-binding cassette transporter g8, Abcg8 in CONTROL (n=7) and RUN (n=7). *p<0.05 vs. CONTROL

We have previously reported indications for impaired cholesterol absorption in chow-fed C57BL/6J mice exposed to a voluntary running wheel as the jejunal expression levels of a crucial protein in cholesterol absorption, Npc1l1, and the plasma plant sterol / cholesterol ratio were decreased in running mice [12]. In contrast, we did not observe an effect on jejunal Npc1l1 expression nor on the plasma plant sterol / cholesterol ratio here. In contrast, we show a running-induced increased expression of jejunal Abcg5/8 the heterodimer cholesterol efflux transporter implicated in the excretion of cholesterol from the intestine [30]. Thus, running appears to differentially affect parameters of cholesterol absorption under low dietary cholesterol, normo-cholesterolemic *versus* high dietary cholesterol, hypercholesterolemic conditions. It is also possible that the previously observed running-induced decreases in expression of Npc1l1 upon 2 weeks of running might underlie

transient adaptations in the intestine, which could be modulated further during longer periods of running.

Noteworthy is that we also found improvements in plasma cholesterol levels and plasma lipoprotein profile and thereby reduced atherosclerotic lesion formation in running mice. Similar small improvements in plasma cholesterol levels have previously been reported in swimming[4] and treadmill-running[22] LDLR-deficient mice and may underlie at least part of the antiatherosclerotic effect.

Intriguing is the running-induced improvements in plasma lipoprotein profiles, showing a marked reduction in the apoB-containing lipoprotein particles VLDL and IDL/LDL. Decreased LDL and VLDL levels have been reported for physically active men [31], however, no such descriptions are available in hypercholesterolemic mice. Furthermore, the improved plasma lipoprotein profiles parallel the running-induced increase in hepatic lipoprotein lipase expression levels and the running-induced decrease in hepatic microsomal transfer protein, suggesting an increased lipoprotein clearance and decreased production, respectively. However, what exactly the role of running in lipoprotein clearance and reduced production is and how this relates to the human situation remains to be explored in future studies.

Altogether, the present study shows that voluntary wheel running is a feasible means to decrease atherosclerotic burden in hypercholesterolemic mice and that an enhanced turnover of cholesterol into bile acids might be the underlying mechanism herein.

ACKNOWLEDGEMENTS

Sources of Funding : This work was supported by GECKO (Groningen Expert Center for Kids with Obesity). The authors want to thank Vincent W. Bloks for critical reading of the manuscript.

REFERENCES

1 Ajijola, O. A., Dong, C., Herderick, E. E., Ma, Q., Goldschmidt-Clermont, P. J. and Yan, Z., Voluntary running suppresses proinflammatory cytokines and bone marrow

endothelial progenitor cell levels in apolipoprotein-E-deficient mice, Antioxid Redox Signal, 2009, 11: 15-23.
2 Blumenthal, J. A., Emery, C. F., Madden, D. J., George, L. K., Coleman, R. E., Riddle, M. W., McKee, D. C., Reasoner, J. and Williams, R. S., Cardiovascular and Behavioral Effects of Aerobic Exercise Training in Healthy Older Men and Women, J Gerontol, 1989, 44: M147-157.
3 Napoli, C., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., Dâ€™Armiento, F. P., Crimi, E., Byrns, R. E., Casamassimi, A., Lanza, A., Gombos, F., Sica, V. and Ignarro, L. J., Physical training and metabolic supplementation reduce spontaneous atherosclerotic plaque rupture and prolong survival in hypercholesterolemic mice, Proceedings of the National Academy of Sciences, 2006, 103: 10479-10484.
4 Napoli, C., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., Rossi, L., Guarino, C., Mansueto, G., Di Tuoro, F., Pignalosa, O., De Rosa, G., Sica, V. and Ignarro, L. J., Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice, Proceedings of the National Academy of Sciences of the United States of America, 2004, 101: 8797-8802.
5 Okabe, T. A., Kishimoto, C., Murayama, T., Yokode, M. and Kita, T., Effects of exercise on the development of atherosclerosis in apolipoprotein E-deficient mice, Exp Clin Cardiol, 2006, 11: 276-279.
6 Pellegrin, M., Berthelot, A., Houdayer, C., Gaume, V., Deckert, V. and Laurant, P., New insights into the vascular mechanisms underlying the beneficial effect of swimming training on the endothelial vasodilator function in apolipoprotein E-deficient mice, Atherosclerosis, 2007, 190: 35-42.
7 Pellegrin, M., Miguet-Alfonsi, C., Bouzourene, K., Aubert, J.-F., Deckert, V., Berthelot, A., Mazzolai, L. and Laurant, P., Long-Term Exercise Stabilizes Atherosclerotic Plaque in ApoE Knockout Mice, Medicine & Science in Sports & Exercise, 2009, 41: 2128-2135.
8 Davis, H. R., Jr, Compton, D. S., Hoos, L. and Tetzloff, G., Ezetimibe, a Potent Cholesterol Absorption Inhibitor, Inhibits the Development of Atherosclerosis in ApoE Knockout Mice, Arterioscler Thromb Vasc Biol, 2001, 21: 2032-2038.
9 Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C. and Hobbs, H. H., Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol, J Clin Invest, 2002, 110: 671-680.
10 Marschall, H. U. and Einarsson, C., Gallstone disease, J Intern Med, 2007, 261: 529-542.
11 Grundy, S. M., Ahrens, E. H., Jr. and Salen, G., Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism, J Lab Clin Med, 1971, 78: 94-121.
12 Meissner, M., Havinga, R., Boverhof, R., Kema, I., Groen, A. K. and Kuipers, F., Exercise Enhances Whole-Body Cholesterol Turnover in Mice, Med Sci Sports Exerc.
13 Arca, M., Montali, A., Ciocca, S., Angelico, F. and Cantafora, A., An improved gas-liquid chromatographic method for the determination of fecal neutral sterols, J Lipid Res, 1983, 24: 332-335.
14 Setchell, K. D., Lawson, A. M., Tanida, N. and Sjovall, J., General methods for the analysis of metabolic profiles of bile acids and related compounds in feces, J Lipid Res, 1983, 24: 1085-1100.
15 Plosch, T., Kok, T., Bloks, V. W., Smit, M. J., Havinga, R., Chimini, G., Groen, A. K. and Kuipers, F., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1, J Biol Chem, 2002, 277: 33870-33877.
16 Mashige, F., Imai, K. and Osuga, T., A simple and sensitive assay of total serum bile acids, Clin Chim Acta, 1976, 70: 79-86.

17 Kuipers, F., Havinga, R., Bosschieter, H., Toorop, G. P., Hindriks, F. R. and Vonk, R. J., Enterohepatic circulation in the rat, Gastroenterology, 1985, 88: 403-411.
18 Windler, E., Zyriax, B. C., Kuipers, F., Linseisen, J. and Boeing, H., Association of plasma phytosterol concentrations with incident coronary heart disease Data from the CORA study, a case-control study of coronary artery disease in women, Atherosclerosis, 2009, 203: 284-290.
19 Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D. and Williams, R. A., Quantitative assessment of atherosclerotic lesions in mice, Atherosclerosis, 1987, 68: 231-240.
20 Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 1959, 37: 911-917.
21 Böttcher, C. S. F., van Gent, C. M. and Fries, C., A rapid and sensitive sub-micro phosphorus determination., Anal Chim Acta, 1961, 24: 203-204.
22 Meilhac, O., Ramachandran, S., Chiang, K., Santanam, N. and Parthasarathy, S., Role of Arterial Wall Antioxidant Defense in Beneficial Effects of Exercise on Atherosclerosis in Mice, Arterioscler Thromb Vasc Biol, 2001, 21: 1681-1688.
23 Shimada, K., Kishimoto, C., Okabe, T. A., Hattori, M., Murayama, T., Yokode, M. and Kita, T., Exercise training reduces severity of atherosclerosis in apolipoprotein E knockout mice via nitric oxide, Circ J, 2007, 71: 1147-1151.
24 Charach, G., Rabinovich, P. D., Konikoff, F. M., Grosskopf, I., Weintraub, M. S. and Gilat, T., Decreased fecal bile acid output in patients with coronary atherosclerosis, J Med, 1998, 29: 125-136.
25 Hulzebos, C. V., Wolters, H., Plosch, T., Kramer, W., Stengelin, S., Stellaard, F., Sauer, P. J., Verkade, H. J. and Kuipers, F., Cyclosporin a and enterohepatic circulation of bile salts in rats: decreased cholate synthesis but increased intestinal reabsorption, J Pharmacol Exp Ther, 2003, 304: 356-363.
26 Liu, Y., Havinga, R., FR, V. D. L., Boverhof, R., Sauer, P. J., Kuipers, F. and Stellaard, F., Dexamethasone exposure of neonatal rats modulates biliary lipid secretion and hepatic expression of genes controlling bile acid metabolism in adulthood without interfering with primary bile acid kinetics, Pediatr Res, 2008, 63: 375-381.
27 Yiamouyiannis, C. A., Martin, B. J. and Watkins, J. B., 3rd, Chronic physical activity alters hepatobiliary excretory function in rats, J Pharmacol Exp Ther, 1993, 265: 321-327.
28 Vieira, V. J., Valentine, R. J., Wilund, K. R., Antao, N., Baynard, T. and Woods, J. A., Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice, Am J Physiol Endocrinol Metab, 2009, 296: E1164-1171.
29 Richard, D. and Trayhurn, P., Effect of exercise training on the rates of fatty acid synthesis in mice, Can J Physiol Pharmacol, 1984, 62: 695-699.
30 Schmitz, G., Langmann, T. and Heimerl, S., Role of ABCG1 and other ABCG family members in lipid metabolism, J. Lipid Res., 2001, 42: 1513-1520.
31 Wood, P. D., Physical activity, diet, and health: independent and interactive effects, Med Sci Sports Exerc, 1994, 26: 838-843.

CHAPTER 7

Beneficial effects of bile acid sequestration and voluntary wheel running on cholesterol turnover and atherosclerosis in hypercholesterolemic mice

M. Meissner, H. Wolters, R.A. de Boer, R.Havinga, R. Boverhof, V.W. Bloks, F. Kuipers, A.K. Groen

Submitted

ABSTRACT

Introduction Bile acid sequestrants (BAS) are cholesterol-lowering agents that have been demonstrated to decrease incidence in cardiovascular events and regression of atherosclerotic plaque formation in clinical trials. However, little is known about the effect of BAS on atherosclerotic lesion formation. Recently, we showed that voluntary running wheel activity (RUN) decreases atherosclerotic lesion size in hypercholesterolemic mice. Lifestyle and pharmacological strategies are often co-prescribed, thus we questioned the effect of BAS treatment alone and in combination with RUN on cholesterol metabolism, heart function and atherosclerotic lesion size in hypercholesterolemic mice.

Methods Male Ldlr-deficient mice remained either sedentary (CONTROL), were treated with 2% (wt/wt in diet) Colesevelam HCl (BAS), had access to a voluntary running wheel (RUN), or were exposed to BAS and RUN (BAS RUN). All groups were fed a high cholesterol diet for 12 weeks.. At 12 weeks of intervention, feces, bile and plasma were collected to determine fecal, biliary and plasma parameters of cholesterol metabolism. Atherosclerotic lesion size was determined in the aortic arch and heart function by echocardiography.

Results BAS RUN ran more than RUN (6.4 ± 1.4 vs. 3.5 ± 1.0 km/day, $p<0.05$). BAS and BAS RUN displayed ~3-fold reductions in plasma cholesterol levels ($p<0.001$), ~2.5-fold increases in fecal neutral sterol ($p<0.001$) and bile acid ($p=0.01$) outputs, decreases in biliary secretions of cholesterol (~6-fold, $p<0.0001$), bile acids and phospholipids (both ~2-fold, $p<0.001$) vs. CONTROL while no effects were observed in RUN. Compared to CONTROL, atherosclerotic lesion size decreased by 78% in both BAS and BAS RUN, ($p<0.0001$).

Conclusion BAS reduce atherosclerosis in Ldlr-deficient mice, coinciding with a switch from cholesterol accumulation to cholesterol loss. RUN slightly modulated atherosclerotic lesion formation but the combination of BAS and RUN had no clear additive effects in this respect.

INTRODUCTION

Atherosclerosis is a complex vascular disease, which is initiated by abnormalities in systemic parameters, such as elevated circulating lipid and lipoprotein levels, leading to inflammation of the vascular wall. Cholesterol has a central role in the pathogenesis of atherosclerosis, excess cholesterol in vascular macrophages leads to formation of atherosclerotic plaques. Strategies that increase the fecal cholesterol excretion as neutral sterols or bile acids, i.e., accelerated whole body cholesterol turnover, improve the pro-atherogenic state by modulating plasma lipid content and thus represent efficient strategies in the amelioration of atherosclerosis.

Fecal excretion of cholesterol as bile acids quantitatively represents a major route of cholesterol removal from the body. Bile acids are synthesized from cholesterol exclusively in the liver and enter the intestinal lumen after a meal. Bile acids are important for the emulsification and absorption of dietary fats in the intestine [1]. .About 95% of the bile acids is reabsorbed from the terminal ileum, transported back to the liver for resecretion into bile (enterohepatic circulation). However, the fraction of bile acids that escapes reabsorption is lost in feces and constitutes an important part of cholesterol turnover, since fecal bile acid loss is compensated for by *de novo* synthesis from cholesterol to maintain the bile acid pool size [2-4]. In addition to their role in lipid uptake, bile acids have emerged as important metabolic regulators of glucose, lipid and energy metabolism [5], which are all implicated in the pathogenesis of atherosclerosis.

Physical activity has long been known as a beneficial strategy for cardiovascular risk reduction [6-15]. We have recently shown that voluntary wheel running enhances cholesterol turnover into bile acids in healthy chow-fed mice [16] as well as in hypercholesterolemic atherosclerotic mice (Chapter 6). Furthermore, the running induced increase in fecal bile acid secretion coincided with a reduction in atherosclerotic lesion size development in hypercholesterolemic running mice (Chapter 6), demonstrating that physical activity beneficially modulates atherosclerosis by enhancing cholesterol turnover in mice. However, despite the recognized benefits of physical activity in the prevention and management of

cardiovascular diseases, regular physical activity programs are frequently reported to be underutilized, adherence to these programs proofs poor or engagement in physical activity is not frequent enough [17, 18]. Moreover, physical activity will not result in adequate improvements in individuals presenting with more aggressive cardiovascular derangements. In these situations, pharmacological intervention becomes necessary and is prescribed either alone or in combination with physical activity.

Bile acid sequestrants (BAS) have long been utilized for improving hypercholesterolemia [19]. Moreover, like physical activity, BAS induce an enhanced cholesterol turnover. BAS are positively charged indigestible resins. In the intestinal lumen they bind to negatively charged bile acids forming non-absorbable complexes, thus increasing fecal bile acid excretion which subsequently stimulates the liver to convert cholesterol into bile acids. A compensatory increase in hepatic LDL receptor activity subsequently clears LDL cholesterol from the circulation, thereby reducing LDL cholesterol levels [19, 20]. Additionally, human studies have shown that colestipol and cholestyramine, two older generation BAS [19], induced reductions in total and LDL cholesterol that were not only accompanied by reductions in relative coronary heart disease death [21] but also by increased regression and decreased progression in coronary artery lesion [22-26]. Colesevelam HCL is a more recent BAS that was specifically engineered for more specificity [19]. However, its effects on atherosclerosis development have not been reported so far.

Thus, the aim of this study was to evaluate the effects of BAS-induced disruption of bile acid metabolism on parameters of cholesterol metabolism, atherosclerosis development and parameters of heart function in hypercholesterolemic mice. Pharmacological intervention and lifestyle changes are often co-prescribed in cardiovascular disease risk management. Therefore, we also investigated whether a combination treatment of BAS and voluntary wheel running would result in additional benefits.

MATERIALS AND METHODS

All experiments were approved by the Animal Care and Use Committee of the University of Groningen, The Netherlands. The University of Groningen is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

Animals, voluntary cage-wheel exercise and BAS treatment

Seventy-four 5 week old male LDLR deficient ((B6.129S7-*Ldlr*^{tm1Her}/J**)** mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were singly housed in a cage (47 x 26 x 14.5 cm) in a temperature-controlled room with a 12:12 light-dark cycle and had *ad libitum* access to standard commercial pelleted laboratory chow (RMH-B, ABDiets, Woerden, the Netherlands) and water. At 8 weeks of age, all mice were switched to a western diet (0.25% cholesterol, 16% fat, Purified Western Diet, 4021.06, ABDiets, Woerden, The Netherlands) and randomly selected to either of the following treatment groups: Sedentary on western-type diet only (CONTROL, n=17), BAS-treatment (BAS, n=17), western-type diet exposed to a voluntary running wheel (RUN, n=20) and BAS exposed to a voluntary running wheel (BAS RUN, n=20). All treatments lasted for 12 weeks. BAS-treated mice were fed the western diet supplemented with 2% (wt/wt) Colesevelam HCL (Daiichi Sankyo, Inc., Parsippany, NJ, USA). Throughout the study, mice had *ad libitum* access to food and water.

The voluntary running wheel set-up utilized has previously been described [27]. Briefly, the cage of RUN mice was equipped with a hamster-sized metal cage wheel with a diameter of 11 cm which was fitted with a cycle computer containing a digital magnetic counter (Art NO.: K-13-TL SET-P3-NL, Xiron, Netherlands). Each morning, total distances ran was recorded. Once a week, mice were weighed and food intake was recorded. Exposing mice to a voluntary running wheel for two weeks has previously been shown to result in cardiac and skeletal muscle

adaptations consistent with those of endurance exercise [27]. Six mice that had access to a voluntary running wheel were excluded from all analyses because they showed no activity on the running wheel.

Experimental procedures

To examine the effect of BAS treatment and the combination of BAS and voluntary wheel running on cholesterol and bile acid metabolism fecal, plasma, biliary, hepatic and intestinal parameters were collected at the endpoint of the experiment after 12 weeks of CONTROL, RUN, BAS and BAS RUN i.e., at 20 weeks of age.

Heart Function

Mice were subjected to transthoracal echocardiography as described [28]. Mice were anaesthetized with 2% isoflurane and body temperature was maintained by placing the mouse on a heating pad (Harvard apparatus). We used a Vivid 7 machine (GE Healthcare, Diegem, Belgium) equipped with special rodent software and a 14 MHz transducer. Cardiac dimensions were measured using parasternal short-axis view and M-mode tracings to determine end-diastolic and end-systolic LV internal diameter, posterior wall thickness (LVPW), and interventricular septal thickness (IVS). Left ventricular mass was calculated as described [29]: LVmass = 1.05 ([LVIDd + LVPWd + IVSd]3—[LVIDd]^3) gram. The cardiac output was calculated by measuring the left ventricular outflow tract (LVOT) diameter and measuring a PW doppler signal over the LVOT (using the formula: LVOT radius2 x π (3.14) LVOT VTI x Heart rate).

Fecal Parameters

Forty-eight hour feces productions were collected at 4 weeks and 12 weeks of treatments and food intake was monitored. Feces were dried, weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total bile acids by an enzymatic fluorimetric assay [30]. Neutral sterols were determined according to Setchell et al. [31]. Dietary cholesterol balance was calculated from the difference of dietary intake and fecal excretion.

Determination of biliary parameters of cholesterol and bile acid metabolism

After 12 weeks of interventions, 8 mice of all groups underwent a gallbladder cannulation for collection of bile [32]. Briefly, mice were anaesthetized by intraperitoneal injection with Hypnorm ® (1 ml·kg-1) and diazepam (10mg·kg-1). During the 30 min bile collection period, mice were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1g·ml-1 for bile. Bile was stored at -20°C until analysis. Total biliary bile acids were determined by an enzymatic fluorimetric assay[33]. Biliary cholesterol and phospholipids levels were measured as described by Kuipers [34].

Determination of plasma markers of cholesterol metabolism

Immediately after the bile collection, blood was collected *via* the abdominal aorta. Plasma was collected by centrifugation and stored at -20°C until analyzed. Plasma total cholesterol, free cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially available assay kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). We analyzed plasma plants sterols relative to plasma cholesterol levels as marker of intestinal cholesterol absorption in pooled plasma samples of each group. Plasma plant sterol (campesterol and sitosterol) concentrations were determined by gas chromatography, as described by Windler et al.[35]. Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium).

Tissue Collection

Mice were opened immediately after blood collection. After dissecting the vena cava, a syringe containing PBS was inserted at the apex of the heart for cleaning. Subsequently the liver was excised, weighed and snap frozen in liquid nitrogen. The small intestine was excised, flushed with ice cold PBS (4°C) and divided into three sections of equal lengths and subsequently snap-frozen in liquid nitrogen. Liver and intestine were stored at -80ºC for later biochemical analysis and RNA

isolation. After collection of all organs the heart was flushed with 1% formalin fix, excised and stored in 1% formalin for no longer than 24 hours. Then hearts were cut in an angle eventually revealing the aortic sinus, embedded in Tissue Tec (Sakura Finetek Europe B.V., Zoeterwoude, NL) and stored in -80ºC.

Determination of atherosclerotic lesion size.

Frozen sections from the aortic sinus were prepared according to Paigen *et al* [36]. Surface lesion area was measured after Oil Red O staining by computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 video camera. At least 5 sections per mouse were examined for each staining.

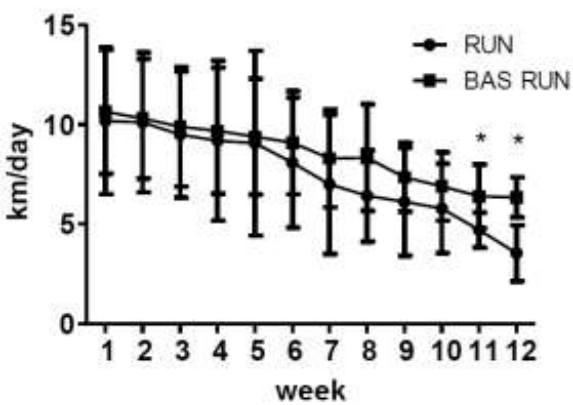


Figure 1. Voluntary running wheel activity during the 12 week running wheel exposure in western-type diet fed mice (RUN) and BAS-treated mice (BAS RUN). Data was statistically analyzed comparing each week to week 1 separately utilizing the Wilcoxon signed-rank-test. Data are expressed as means ± SD, *p<0.05 vs. 1 week

Determination of hepatic lipids

Hepatic lipids were determined after extraction according to Bligh and Dyer [37] using the same kits as for plasma lipids. Phospholipids were determined in all tissues according to Böttcher et al[38].

RNA isolation and PCR procedures

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers' protocol. cDNA was produced as described by Plösch and coworkers [32]. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad

IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to β-actin for liver and to cyclophyllin and 36B4 for intestine.

Statistics

Multiple group comparisons were done by the Kruskal-Wallis H test, followed by Posthoc Conover test, using Bright Stat software [39] unless stated otherwise. All data are expressed as means ± SD. A *P*-value of <0.05 was accepted as statistically significant.

Table 1. Biometrical data. Values represent mean ± SD at 12 weeks of running in CONTROL, RUN, BAS and BAS RUN, (n=8 for each group), *p<0.05 vs. CONTROL, †p<0.05 vs. BAS

	CONTROL	RUN	BAS	BAS RUN
Body weight (g)	30.2 ± 3.1	28.6 ± 0.9	26.0 ± 0.6 *	26.1 ± 1.0 *
Liver weight (g)	1.43 ± 0.14	1.36 ± 0.10	1.26 ± 0.05*	1.3 ± 0.08
Liver / body weight (%)	4.7 ± 0.1	4.8 ± 0.3	4.8 ± 0.1	4.9 ± 0.3
Food intake (g / day)	3.2 ± 0.2	3.9 ± 0.4*	4.1 ± 0.3*	5.0 ± 0.5*†

RESULTS

Effect of BAS treatment on morphometric parameters and running wheel activity

Despite an increased food intake compared to control mice, BAS-treated running and sedentary mice both displayed a ~14% decrease in body weight that was paralleled by a decrease in liver weights in BAS mice, thus resulting in absence of a net effect on the liver-to-body weight ratio (Table 1). All mice exposed to a voluntary running wheel progressively ran less during the 12 week running wheel intervention. While a daily average running distance of ~10 km was observed at the beginning of the experiment, it dropped to 6.4 km/day in BAS-treated mice and even to 3.5 km/day in non-BAS-treated running mice (Figure 1). Furthermore, we observed a significant decrease in running wheel activity in non-BAS treated mice

compared to BAS-treated mice starting at 11 weeks of running wheel exposure (Figure 1).

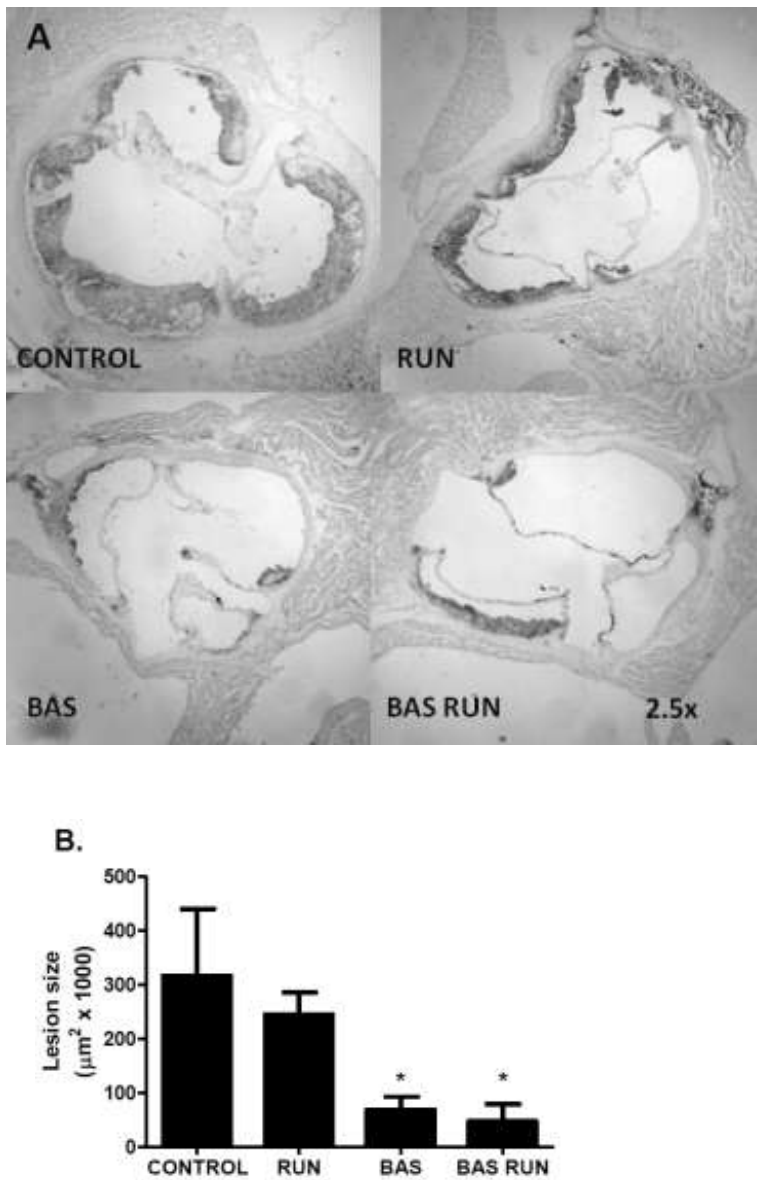


Figure 2. BAS treatment reduces atherosclerotic lesion size. (A) Representative morphological sections of aortic sinus stained with Oil Red Oil of CONTROL, RUN, BAS and BAS RUN mice, (B) Quantification of lesion size in aortic sinus of CONTROL (n=9), RUN (n=9), BAS (n=10) and BAS RUN (n=7) mice at 12 weeks of intervention. *p<0.05 vs. CONTROL

BAS treatment decreases atherosclerotic lesion size development in LDLR-deficient mice

First, we investigated whether 12 weeks of BAS treatment was beneficial in reducing atherosclerotic lesion size development in atherosclerosis-prone LDLR-deficient mice and whether a combination with voluntary running wheel exercise would have additional effects. Indeed, quantification of atherosclerotic lesion size in aortic sinus showed a 78% reduction in BAS-treated mice compared to control mice (Figure 2). The combination treatment of BAS and voluntary wheel exercise yielded no additional effects on reducing atherosclerotic lesion size. Running mice not treated with BAS, showed a 23% reduction in atherosclerotic lesion size compared to control mice. Atherosclerosis can over time lead to hemodynamic impairments in the heart and thus lead to acute myocardial events. Impairments in cardiac function have previously been reported for 36-week old LDLR-deficient mice fed a western diet [40, 41]. Thus, we evaluated whether the BAS mediated reduction in atherosclerotic lesion size would impact on cardiac function in the 20-week old LDLR-deficient mice of this study. However, no differences were observed in left ventricular mass, percent of fractional shortening, heart rate or cardiac output upon BAS and combination treatment (Table 2).

Table 2. Cardiac function. Values represent mean ± SD at 12 weeks of running in CONTROL, RUN, BAS and BAS RUN, (n=8 for each group), *p<0.05 vs. CONTROL, †p<0.05 vs. BAS; LV = left ventricular mass

	CONTROL	RUN	BAS	BAS RUN
LV mass (mg)	95.2 ± 16.5	96.3 ± 24.7	88.4 ± 17.2	86.1 ± 15.4
% Fractional Shortening	43 ± 4	43 ± 3	45 ± 4	47 ± 5
Heart Rate (beats per min)	443 ± 49	415 ± 56	395 ± 41	417 ± 26
Cardiac Output (ml/min)	31.7 ± 4.7	28.5 ± 5.0	29.4 ± 4.9	28.5 ± 5.8

BAS treatment beneficially affects lipid metabolism in LDLR-deficient mice

Elevated lipid levels are key in the development of atherosclerosis. Thus, we evaluated the effect of BAS treatment alone and in combination with voluntary wheel running on plasma lipid levels, plasma lipoprotein profiles as well as on

hepatic parameters involved in lipoprotein and lipid metabolism. BAS treated mice displayed significant reductions in plasma levels of total cholesterol (2.8-fold), esterified cholesterol (2.5-fold), free cholesterol (3.7-fold) and triglycerides (3.9-fold) compared to control mice (Table 3), while no additional improvements were observed in the combination treatment. Importantly, we found improved plasma lipoprotein profiles with reduced levels of VLDL- and LDL-sized lipoproteins in all BAS treated mice compared to control mice (Figure 3).

Further beneficial effects of BAS treatment were observed on hepatic lipid storage. BAS treated mice displayed 1.8-fold reductions in hepatic triglyceride stores compared to control mice (Table 3), which was paralleled by a 40% reduction in hepatic expression of the lipogenic gene Srebp1c (Table 4). The combination treatment of BAS and voluntary wheel running led to further reductions in hepatic triglyceride stores and hepatic Srebp1c levels compared to BAS treatment alone. Moreover, all BAS treated mice displayed reductions in hepatic total cholesterol (2.2-fold), free cholesterol (1.5-fold) and esterified cholesterol contents (3.8-fold) compared to control mice. Despite the BAS-induced decrease in hepatic cholesterol stores, we found increased hepatic levels of Hmgcr (4.3-fold induction),

Table 3. Plasma and liver lipids. Values represent mean ± SD at 12 weeks of running in CONTROL, RUN, BAS and BAS RUN, (n=8 for each group), *p<0.05 vs. CONTROL, †p<0.05 vs. BAS

	CONTROL	RUN	BAS	BAS RUN
Plasma Lipids (mM)				
Total cholesterol	30.4± 3.4	30.2 ± 4.3	10.7 ± 1.2*	12.5 ± 3.0*
Free \cholesterol	10.8 ± 1.0	10.8 ± 1.5	2.9 ± 0.6*	3.8 ± 1.4*
Cholesterol esters	19.6 ± 2.4	19.4 ± 2.9	7.8 ± 0.7*	8.7 ± 1.7*
Triglycerides	11.9 ± 1.3	10.9 ± 1.3	3.0 ± 0.5*	3.3 ± 1.2*
Liver Lipids (nmol/mg liver)				
Triglycerides	40.6 ± 7.7	23.3 ± 5.5*	23.4 ± 3.4*	13.6 ± 1.2 *†
Total cholesterol	18.0 ± 3.3	21.1 ± 4.0	8.2 ± 0.8 *	8.1 ± 0.8 *
Free cholesterol	8.2 ± 0.7	8.2 ± 0.7	5.6 ± 0.4 *	5.8 ± 0.3 *
Cholesterol esters	10.0 ± 3.1	13.0 ± 3.4	2,6 ± 0.5 *	2.3 ± 0.5 *
Phospholipids	26.7 ± 1.3	28.2 ± 1.3*	26.1 ± 3.1	27.1 ± 1.3

Effects of BAS treatment on sterol metabolism

which encodes the rate-limiting enzyme in cholesterol biosynthesis, and *Srebp2* (2-fold induction), a protein implicated in control of cholesterol synthesis upon cellular cholesterol depletion, in BAS-treated mice (*Table 4*). Collectively, these data show that BAS provokes favorable changes in lipid metabolism.

Next, we assessed whether the BAS-induced beneficial changes in atherosclerotic lesion size and the improvements in plasma and hepatic lipid levels were accompanied by BAS-induced modulation of cholesterol and bile acid metabolism.

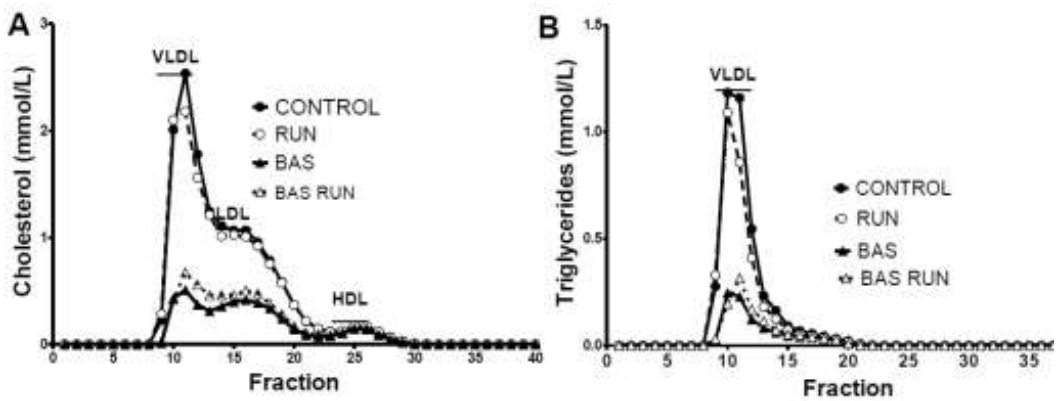


Figure 3. FPLC separation of plasma lipoproteins of CONTROL, RUN, BAS and BAS RUN. Plasma of 8 mice per group was pooled and separated by fast protein liquid chromotography (FLPC) on a Superose 6 column. Cholesterol (A) and triglyceride (B) content was measured in each fraction. VLDL = very low density lipoprotein, LDL = low densitiy lipoprotein and HDL = high density lipoprotein

.

BAS treatment increased fecal sterol output in LDLR-deficient mice

We first assessed fecal parameters of cholesterol and bile acid metabolism at 12 weeks of the intervention. BAS-treated mice had significantly higher neutral sterol and bile acid excretions (2.3-fold and 4.2-fold, respectively; Table 5). As fecal bile acid excretion is reflective of de novo synthesis, BAS treated mice displayed an increased de novo bile acid synthesis. This was paralleled by a BAS-induced increase in hepatic expression of genes encoding bile acid synthesizing proteins Cyp7a1, Cyp8b1 and Cyp27 (Table 5) and by decreased ileal expression of

fibroblast growth factor 15 (Fgf15), a gene which acts to inhibit bile acid synthesis (Table 5).

BAS treatment promotes a negative cholesterol balance

Because accumulation of excess cholesterol in vascular macrophages leads to the formation of atherosclerotic plaques, we then calculated the whole body cholesterol balances. The difference between dietary cholesterol intake and fecal cholesterol excretion was positive in non-BAS treated mice, (Table 5) demonstrating cholesterol accumulation in the body. However, the cholesterol balance was markedly decreased and became negative in BAS treated mice, even more in BAS-only treated mice than in mice receiving the combination treatment, showing that BAS treated mice excreted their accumulated cholesterol and when in a new steady state compensated by an increase in de novo synthesis (Table 5).

BAS treatment decreased biliary sterol secretions

Subsequently, we assessed biliary parameters. Mice were subjected to gallbladder cannulation for collection of hepatic bile at 12 weeks of interventions. BAS-treated mice had a 11% decreased bile flow, and major reductions in biliary secretions of cholesterol (73% decreased, *Table 5*), bile acids (55% decreased, Table 5) and phospholipids (50% decreased, data not shown) compared to control mice. The BAS-induced decrease in biliary cholesterol secretion was paralleled by a decrease in hepatic ATP-binding cassette transporter protein g5 and a slight, albeit not significant, reduction in g8 (Table 5), which both act to transport cholesterol across the canalicular membrane into the bile. BAS had no effect on hepatic Bsep (Abcb11) expression, which acts to transport bile acids across the canalicular membrane (Table 5). The combination treatment did not result in differential or stronger effects on fecal and biliary parameters than BAS treatment alone.

BAS treatment decreased bile acid reabsorption

From the fecal and biliary data we calculated other parameters of *in vivo* bile acid and cholesterol turnover. We found the daily reabsorption of bile acids dramatically decreased upon BAS treatment (96 $\pm$ 1 % in CONTROL vs. 50 $\pm$ 25% in BAS and

Table 4. Hepatic and intestinal genes of cholesterol and bile acid metabolism.
Hepatic and intestinal mRNA expression levels for sterol-responsive-binding-protein 1c (Srepb1c),3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr), sterol-responsive- binding protein 2 (Srebp2), ATP-binding cassette transporter g5 (Abcg5) and g8 (Abcg8), cholesterol 7 alpha- hydroxylase (Cyp7a1), sterol 12-alpha-hydroxylase (Cyp8b1), sterol 27-hydroxylase (Cyp27), bile salt export pump (Bsep), ATP-binding cassette transporter ABCA1 (Abca1),apical sodium dependent bile aicd transporter (Asbt), ileal bile acid binding protein (Ibabp), fibroblast growth factor 15 (Fgf15) in CONTROL (n=8), RUN (n=8), BAS (n=8) and BAS RUN (n=8); Hepatic values are relative to β-actin, Intestinal values are relative to cyclophyllin and 36B4 all values represent mean ± SD, *p<0.05 vs. CONTROL, †p<-0.05 vs. BAS

	CONTROL	RUN	BAS	BAS RUN
Liver				
Lipogenessis				
Srebp1c	1.0 ± 0.2	0.7 ± 0.2*	0.6 ± 0.1*	0.5 ± 0.1*†
Cholesterol Synthesis				
Hmgcr	1.0 ± 0.3	0.6 ± 0.2*	4.3 ± 1.0*	5.0 ± 1.0*
Srebp2	1.0 ± 0.1	0.9 ± 0.2	2.0 ± 0.3*	1.9 ± 0.2*
Cholesterol Excretion				
Abcg5	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.1*	0.7 ± 0.2*
Abcg8	1.0 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.8 ± 0.2
Bile Acid Metabolism				
Cyp7a1	1.0 ± 0.6	0.7 ± 0.4	3.8 ± 1.6*	5.3 ± 2.0*
Cyp8b1	1.0 ± 0.2	0.8 ± 0.2	2.1 ± 0.4*	2.1 ± 0.3*
Cyp27	1.0 ± 0.2	0.9 ± 0.2	1.3 ± 0.2*	1.3 ± 0.2*
Bsep	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.1*
Intestine				
Cholesterol Metabolism				
Jejunal Abcg5	1.0 ± 0.2	1.3 ± 0.2	0.7 ± 0.1*	0.7 ± 0.2*
Jejunal Abcg8	1.0 ± 0.3	1.3 ± 0.2	0.6 ± 0.1*	0.6 ± 0.2*
Jejunal Abca1	1.0 ± 0.2	0.8 ± 0.2	0.3 ± 0.1*	0.3 ± 0.1*
Bile Acid Metabolism				
Ileal Asbt	1.0 ± 0.4	1.0 ± 0.4	2.6 ± 0.9*	2.3 ± 0.4*
Ileal Ibabp	1.0 ± 0.3	1.1 ± 0.2	0.6 ± 0.1*	0.6 ± 0.1*
Ileal Fgf15	1.0 ± 0.5	1.8 ± 0.9	0.02 ± 0.01*	0.02 ± 0.02*

41 ± 30% in BAS RUN). Despite the decreased bile acid reabsorption, ileal expression of the apical sodium dependent bile acid transporter (Asbt, Slc10a2), was induced more than 2-fold in BAS-treated mice, however, expression of ileal bile acid binding protein (Ibabp, Fabp6), which is downstream of Asbt and

facilitates intracellular bile acid transport in the ileocyte, was decreased with BAS treatment (Table 4).

BAS treatment increased cholesterol secretion from non-biliary compartment

As the fecal cholesterol loss was by far higher than the sum of biliary and dietary cholesterol input upon BAS treatment, we calculated by how much a non-hepatobiliary cholesterol excretion could account for this massive BAS-induced cholesterol loss. We have previously determined the fractional cholesterol absorption to be 38.3 ± 11% in LDLR-deficient mice fed the western-type diet for 12 weeks (unpublished data) and thus used this value for non-BAS treated mice. We were not able to assess cholesterol absorption upon BAS treatment. Yet, In light of the by ~6-fold decreased plasma plant sterol / cholesterol ratio (Table 5) upon BAS treatment, the rate of cholesterol absorption must have decreased considerably. We therefore calculated the non-hepatobiliary cholesterol excretion for BAS treated mice using a cholesterol absorption of 0. This would also yield the lowest possible non-hepatobiliary cholesterol excretion and thus prevent overestimation. The calculated non-hepatobiliary cholesterol excretion values were more than ~2.3-fold higher in BAS-treated mice, suggesting that this non-hepatobiliary route of cholesterol excretion is enhanced under BAS. Jejunal expressions of ATP-cassette binding transporters g5 and g8 and Abca1, proteins known to promote cholesterol transport from the enterocyte back into the intestinal lumen for elimination into feces, were, however, decreased in BAS-treated mice (Table 4).

DISCUSSION

In the present study, we describe the effects of disrupting the enterohepatic circulation of bile acids by Colesevelam HCL, a new generation bile acid sequestrant (BAS) in an established mouse model of atherosclerosis. Since lifestyle interventions, such as physical activity, are often co-prescribed to pharmacological intervention, we furthermore evaluated whether a combination treatment of BAS and voluntary wheel running would yield additional benefits over

Table 5. Parameters of cholesterol and bile acid metabolism.
This table represents measured and calculated values of fluxes involved in the enterohepatic system and parameters of cholesterol metabolism. Values for bile acid synthesis were assumed to equal the measured values for bile acids excreted via the feces. Daily bile acid reabsorption was calculated by the difference in measured bile acids secreted in the bile and feces. Dietary cholesterol balance was calculated by the difference between dietary cholesterol intake and fecal cholesterol excretion. Fractional cholesterol absorption in non-BAS-treated mice was set to 38%, a value determined by us previously utilizing the dual isotope dilution technique in LDLR-deficient mice fed the western diet for 12 weeks (unpublished data). Fractional cholesterol absorption was set to 0% for BAS-treated mice taking into account the decrease in plasma plant sterols / cholesterol ratio which is indicative of a decreased cholesterol absorption and taking account that lower values of absorption give lower non-hepatobiliary cholesterol excretion values; thus non-hepatobiliary cholesterol excretion values calculated represent the lowest possible values and thus might be underestimated for the BAS-treated groups. Non-hepatobiliary cholesterol excretion was calculated by the difference between the sum of fecal and absorbed cholesterol and the sum of dietary and biliary cholesterol Values represent mean ± SD at 12 weeks of interventions for CONTROL (n=6), RUN (n=8), BAS (n=8) and BAS RUN (n=8); *p<0.05 vs. CONTROL

	Control	RUN	BAS	BAS RUN
Bile flow (ul/min/100gBW)	5.47 ± 0.90	6.00 ± 0.49	4.86 ± 0.62*	5.12 ± 0.82
Bile Acids (μmol/day/100gBW)				
Bile	210 ± 22	250 ± 105	94 ± 37*	106 ± 37*
Feces	9 ± 2	12 ± 4	40 ± 4*	53 ± 14*
Synthesis	9 ± 2	12 ± 4	40 ± 4*	53 ± 14*
Reabsorption	201 ± 22	238 ± 106	55 ± 39*	53 ± 51*
Absorption (%)	96 ± 1	94 ± 4	50 ± 25*	41 ± 30*
Feces (%)	4 ± 1	6 ± 4	50 ± 25*	59 ± 30*
Cholesterol (μmol/day/100gBW)				
Dietary intake	83 ± 4	97 ± 8*	135 ± 7*	156 ± 14*
Bile	2.5 ± 0.2	1.9 ± 0.8	0.4 ± 0.4*	0.7 ± 0.6*
Dietary cholesterol balance	21 ± 6	17 ± 5	-36 ± 10*	-22 ± 14*
Feces	62 ± 8	79 ± 8	171 ± 12*	178 ± 19*
Absorption	38 ± 11	38 ± 11	0 ± 0*	0 ± 0*
Non-hepatobiliary cholesterol excretion	16 ± 10	19 ± 6	36 ± 7*	22 ± 15*
Plasma plant sterol/ cholesterol (μmol/mmol)	1.5 ± 0.4	1.3 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1*

BAS treatment alone. We were able to show, for the first time, that treatment with Colesevelam HCL indeed provokes specific changes in cholesterol metabolism, specifically switching from a positive to a negative cholesterol balance and increasing cholesterol removal *via* non-hepatobiliary route. These favourable BAS-induced modulations translated into improved plasma as well as liver lipid metabolism and ultimately into a dramatic (78%) reduction in atherosclerotic lesion size development in western-type diet fed, hypercholesterolemic LDLR deficient mice. Moreover, we show that a combination treatment of BAS and physical activity had no additional benefits herein. Physical exercise alone modestly reduced lesion size (23%). Surprisingly, heart function was normal in all groups studied.

Our data clearly show that under severe hypercholesterolemia as in LDLR-deficiency, BAS treatment is much more effective in decreasing atherosclerotic lesion size development than voluntary wheel running alone, which is in line with the degree of metabolic changes induced by either intervention. Intriguingly, running mice not treated with BAS ran ~2-times less than running mice treated with BAS during the last two weeks of the experiment. To our surprise, they also ran significantly less than LDLR deficient mice fed a western-type diet in a previous study (Chapter 6). In fact, as running activity of these mice was decreased by half in this experiment compared to our previous one, the improvements in atherosclerotic lesion size development were also decreased by a similar magnitude (44% reduction in atherosclerotic lesion upon running compared to control mice in our previous study to a 23% reduction in this study). In fact calculating the area under the curve for the average distance ran per day for every one of the 12 weeks and plotting this against lesion size for running mice of this and the previous experiment, we found a significant correlation between area under the curve and lesion size (Spearman r= -0.63, *P*=0.044). A dose-response relationship between amount of physical activity and cardiovascular risk reduction has previously been observed [42, 43] and the decrease in physical activity in running only mice observed here is most likely the underlying factor for the discrepancy in findings between this and the previous study. Moreover, this discrepancy in physical activity and cardiovascular risk reduction between our previous study and

the present investigation parallels pitfalls of human physical activity intervention programs when compliance is often too low to result in adequate cardiovascular risk reduction [18, 44, 45] and pharmacological interventions are called upon for efficient treatment.

Our data support the notion that BAS provide an attractive strategy in treating lipid disorders and associated diseases, as monotherapy or as add-on therapy in patients that are intolerant to statins or respond inadequately to statins. Due to their cholesterol-lowering properties, it has been long appreciated that BAS reduce cardiovascular risk, including a reduced incidence of acute myocardial events [26]. Additionally, clinical trials have revealed that cholesteryramine and colestipol, two older generation BAS, increased regression and decreased progression in coronary artery lesion as determined by angiography [22-26]. We demonstrate here, for the first time, that Colesevelam HCL, a more recent and specifically engineered BAS with better side effect profile and efficacy over time [19], substantially reduces atherosclerotic lesion size in hypercholesterolemic mice. Thus, the results of the present study warrant the evaluation of Colesevelam HCL on atherosclerotic lesion size reduction in human trials.

The differences in lesion size between groups did, in contrast to our expectations, not associate with differences in cardiac function in any group of the LDLR-deficient mice. All echocardiographic parameters were comparable to previously published reference values for C57BL/6J and CD1 mice [46, 47]. Previous studies showed impaired heart function in 36-week old LDLR deficient mice [40, 41]. Other reports suggested that LDLR deficient mice are prone to cardiac dysfunction, even on normal diet [48] or on very short-term (15 days) western-type diet [49]. We could not confirm these observations. It may be that our intervention was too short lasting to elicit cardiac ischemia and subsequent damage. Thus, we suspect that the control mice in the present study were too young and atherosclerosis thereby not advanced enough for abnormalities in heart function to manifest. Alternatively, although the LDLR deficient mouse has resemblance with human coronary artery disease, the sequence of events is rather different from man and cardiac dysfunction and heart failure does not seem as prevalent in this mouse model as in

human disease. It thus remains to be determined whether BAS treatment benefits heart function in more advanced atherosclerosis.

Numerous studies have previously shown that BAS-induced interruption of the enterohepatic circulation of bile acids leads to an enhanced hepatic cholesterol demand for bile acid synthesis [50-52]. Our data presented here demonstrate a BAS-induced response for the increased demand for cholesterol biosynthesis in several ways. First, we found a more than ~4-fold induction of the rate-limiting enzyme of cholesterol synthesis, Hmgcr, in BAS-treated mice. This is in line with the increased Hmgcr expression and fractional cholesterol synthesis we recently reported in Colesevelam HCL-treated lean and *db/db* mice on normal chow [50] but also supports an older study showing that cholestyramine increased Hmgcr activity ~6-fold in gallstone patients [51]. Secondly, we found BAS-induced reductions in plasma LDL-sized lipoproteins paralleling earlier human studies using cholestyramine [52, 53]. Moreover, we demonstrate a BAS-induced increase in bile acid synthesis in several ways. First, the BAS-induced more than 4-fold increase in fecal bile acid output is reflective of an increased hepatic synthesis. Second, *via* the BAS-induced increases in hepatic Cyp7a1, Cyp8b1 and Cyp27 mRNA levels, we demonstrate an induction in bile acid synthesizing transcriptional machinery, paralleling our earlier observations of increased bile acid transcriptional machinery and bile acid synthesis rate in BAS treated lean and *db/db* mice on normal chow [50].

Importantly, our data show that 12 weeks of Colesevelam HCL treatment induces a new steady state in the enterohepatic flux of bile acids in hypercholesterolemic atherosclerotic mice. For example within the model we studied, the calculated bile acid reabsorption was reduced to 50% in BAS-treated mice compared to control mice, which is higher than the BAS-induced 30% reduction in bile acid reabsorption observed in lean and *db/db* mice [50], indicating that the effect of BAS on bile acid metabolism is even greater in hypercholesterolemic mice. Additionally, while previous studies report no effects of BAS on biliary lipid secretions [50, 53] and bile acid pool size [50], we observed profound reductions in biliary lipid secretion upon BAS here. These differences might be attributable to duration of treatment, diets or mouse strain studied. While the previous reports concern either Colesevelam HCL supplementation to normal

chow for two weeks in lean and obese diabetic *db/db* mice or 4 week cholestyramine administration to healthy normo-lipidaemic men, we studied the effects of BAS on an extreme model of atherosclerosis after 12 weeks of Colesevelam HCL supplementation to a western-type diet. The decrease in biliary bile acid secretion is likely reflective of a decreased bile acid pool size and indicates that *de novo* bile acid synthesis adapted to a new steady state in BAS treated mice of this experiment.

Additionally, BAS treatment exerted substantial effects on the cholesterol balance. Under steady state conditions the amount of cholesterol from dietary input and endogenous synthesis equals fecal excretion. In this situation there is no accumulation of cholesterol in the body. A positive cholesterol balance leads to accumulation of excess cholesterol within the body which can lead to formation of atherosclerotic plaque. The LDLR-deficient mouse has been long utilized as a murine model for atherosclerosis due to its inability of hepatic LDL-lipoprotein uptake which subsequently results in atherosclerosis. However, the actual whole-body cholesterol balance upon Ldlr deficiency has not been reported. We show that Ldlr deficiency leads to cholesterol accumulation and that BAS treatment is effectively interfering herein leading to a substantial cholesterol loss which coincided with a reduction in atherosclerosis. The involvement of a non-hepatobiliary cholesterol excretion pathway appears to have a major contribution herein. BAS-treated mice displayed a substantial deficit between the fecal sterol excretion and the sum of dietary cholesterol intake and biliary cholesterol secretion. This demonstrates that BAS treatment strongly enhances cholesterol excretion from a non-hepatobiliary route. We calculated the contribution of this non-hepatobiliary cholesterol excretion pathway to be at least 2.3-fold enhanced.

In summary, the present study shows that disruption of the enterohepatic circulation by BAS treatment substantially reduced atherosclerotic lesion size in hypercholesterolemic mice which coincided with profound changes in sterol metabolism, specifically leading to a massive cholesterol loss. A combination treatment of physical activity and BAS had no additive effects. Now that a new generation BAS compound has been developed, with a much better safety and

side-effects profile [54, 55], it may be imperative to re-evaluate the usefulness of BAS in fighting atherosclerosis in humans.

REFERENCES

1 Marschall, H. U. and Einarsson, C., Gallstone disease, J Intern Med, 2007, 261: 529-542.
2 Witztum, J. L., Young, S. G., Elam, R. L., Carew, T. E. and Fisher, M., Cholestyramine-induced changes in low density lipoprotein composition and metabolism. I. Studies in the guinea pig, J Lipid Res, 1985, 26: 92-103.
3 Grundy, S. M., Ahrens, E. H., Jr. and Salen, G., Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism, J Lab Clin Med, 1971, 78: 94-121.
4 Levy, R. I., Fredrickson, D. S., Stone, N. J., Bilheimer, D. W., Brown, W. V., Glueck, C. J., Gotto, A. M., Herbert, P. N., Kwiterovich, P. O., Langer, T., LaRosa, J., Lux, S. E., Rider, A. K., Shulman, R. S. and Sloan, H. R., Cholestyramine in type II hyperlipoproteinemia. A double-blind trial, Ann Intern Med, 1973, 79: 51-58.
5 Kuipers, F., Stroeve, J. H., Caron, S. and Staels, B., Bile acids, farnesoid X receptor, atherosclerosis and metabolic control, Curr Opin Lipidol, 2007, 18: 289-297.
6 Kramsch, D., Aspen, A., Abramowitz, B., Kreimendahl, T. and Hood, W., Reduction of coronary atherosclerosis by moderate conditioning exercise in monkeys on an atherogenic diet, N Engl J Med, 1981, 305: 1483-1489.
7 Ajijola, O. A., Dong, C., Herderick, E. E., Ma, Q., Goldschmidt-Clermont, P. J. and Yan, Z., Voluntary running suppresses proinflammatory cytokines and bone marrow endothelial progenitor cell levels in apolipoprotein-E-deficient mice, Antioxid Redox Signal, 2009, 11: 15-23.
8 Napoli, C., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., Dâ€™Armiento, F. P., Crimi, E., Byrns, R. E., Casamassimi, A., Lanza, A., Gombos, F., Sica, V. and Ignarro, L. J., Physical training and metabolic supplementation reduce spontaneous atherosclerotic plaque rupture and prolong survival in hypercholesterolemic mice, Proceedings of the National Academy of Sciences, 2006, 103: 10479-10484.
9 Pellegrin, M., Alonso, F., Aubert, J.-F., Bouzourene, K., Braunersreuther, V., Mach, F., Haefliger, J.-A., Hayoz, D., Berthelot, A., Nussberger, J., Laurant, P. and Mazzolai, L., Swimming Prevents Vulnerable Atherosclerotic Plaque Development in Hypertensive 2-Kidney, 1-Clip Mice by Modulating Angiotensin II Type 1 Receptor Expression Independently From Hemodynamic Changes, Hypertension, 2009, 53: 782-789.
10 Pellegrin, M., Miguet-Alfonsi, C., Bouzourene, K., Aubert, J.-F., Deckert, V., Berthelot, A., Mazzolai, L. and Laurant, P., Long-Term Exercise Stabilizes Atherosclerotic Plaque in ApoE Knockout Mice, Medicine & Science in Sports & Exercise, 2009, 41: 2128-2135.
11 Blumenthal, J. A., Emery, C. F., Madden, D. J., George, L. K., Coleman, R. E., Riddle, M. W., McKee, D. C., Reasoner, J. and Williams, R. S., Cardiovascular and Behavioral Effects of Aerobic Exercise Training in Healthy Older Men and Women, J Gerontol, 1989, 44: M147-157.
12 Ramachandran, S., Penumetcha, M., Merchant, N. K., Santanam, N., Rong, R. and Parthasarathy, S., Exercise reduces preexisting atherosclerotic lesions in LDL receptor knock out mice, Atherosclerosis, 2005, 178: 33-38.
13 Okabe, T. A., Kishimoto, C., Murayama, T., Yokode, M. and Kita, T., Effects of exercise on the development of atherosclerosis in apolipoprotein E-deficient mice, Exp Clin Cardiol, 2006, 11: 276-279.
14 Pellegrin, M., Berthelot, A., Houdayer, C., Gaume, V., Deckert, V. and Laurant, P., New insights into the vascular mechanisms underlying the beneficial effect of swimming

training on the endothelial vasodilator function in apolipoprotein E-deficient mice, Atherosclerosis, 2007, 190: 35-42.
15 Napoli, C., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., Rossi, L., Guarino, C., Mansueto, G., Di Tuoro, F., Pignalosa, O., De Rosa, G., Sica, V. and Ignarro, L. J., Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice, Proceedings of the National Academy of Sciences of the United States of America, 2004, 101: 8797-8802.
16 Meissner, M., Havinga, R., Boverhof, R., Kema, I., Groen, A. K. and Kuipers, F., Exercise Enhances Whole-Body Cholesterol Turnover in Mice, Med Sci Sports Exerc.
17 Kamiya, A., Ohsawa, I., Fujii, T., Nagai, M., Yamanouchi, K., Oshida, Y. and Sato, Y., A clinical survey on the compliance of exercise therapy for diabetic outpatients, Diabetes Res Clin Pract, 1995, 27: 141-145.
18 Swift, C. S., Armstrong, J. E., Beerman, K. A., Campbell, R. K. and Pond-Smith, D., Attitudes and beliefs about exercise among persons with non-insulin-dependent diabetes, Diabetes Educ, 1995, 21: 533-540.
19 Insull, W. J. M. D., Clinical Utility of Bile Acid Sequestrants in the Treatment of Dyslipidemia: A Scientific Review, Southern Medical Journal, 2006, 99: 257-273.
20 Isaacsohn, J., Hunninghake, D., Schrott, H., Dujovne, C. A., Knopp, R., Weiss, S. R., Bays, H., Crouse, J. R., 3rd, Davidson, M. H., Keilson, L. M., McKenney, J., Korenman, S. G., Dobs, A. S., Stein, E., Krauss, R. M., Maccubbin, D., Cho, M., Plotkin, D. J. and Mitchel, Y. B., Effects of simvastatin, an HMG-CoA reductase inhibitor, in patients with hypertriglyceridemia, Clin Cardiol, 2003, 26: 18-24.
21 The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering, JAMA, 1984, 251: 365-374.
22 Brensike, J. F., Levy, R. I., Kelsey, S. F., Passamani, E. R., Richardson, J. M., Loh, I. K., Stone, N. J., Aldrich, R. F., Battaglini, J. W., Moriarty, D. J. and et al., Effects of therapy with cholestyramine on progression of coronary arteriosclerosis: results of the NHLBI Type II Coronary Intervention Study, Circulation, 1984, 69: 313-324.
23 Blankenhorn, D. H., Nessim, S. A., Johnson, R. L., Sanmarco, M. E., Azen, S. P. and Cashin-Hemphill, L., Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts, JAMA, 1987, 257: 3233-3240.
24 Cashin-Hemphill, L., Mack, W. J., Pogoda, J. M., Sanmarco, M. E., Azen, S. P. and Blankenhorn, D. H., Beneficial effects of colestipol-niacin on coronary atherosclerosis. A 4-year follow-up, JAMA, 1990, 264: 3013-3017.
25 Kane, J. P., Malloy, M. J., Ports, T. A., Phillips, N. R., Diehl, J. C. and Havel, R. J., Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens, JAMA, 1990, 264: 3007-3012.
26 Watts, G. F., Lewis, B., Brunt, J. N., Lewis, E. S., Coltart, D. J., Smith, L. D., Mann, J. I. and Swan, A. V., Effects on coronary artery disease of lipid-lowering diet, or diet plus cholestyramine, in the St Thomas' Atherosclerosis Regression Study (STARS), Lancet, 1992, 339: 563-569.
27 Allen, D. L., Harrison, B. C., Maass, A., Bell, M. L., Byrnes, W. C. and Leinwand, L. A., Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse, J Appl Physiol, 2001, 90: 1900-1908.
28 Wiersma, H., Nijstad, N., Gautier, T., Iqbal, J., Kuipers, F., Hussain, M. M. and Tietge, U. J., Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice, J Lipid Res, 51: 544-553.
29 Troy, B. L., Pombo, J. and Rackley, C. E., Measurement of left ventricular wall thickness and mass by echocardiography, Circulation, 1972, 45: 602-611.
30 Arca, M., Montali, A., Ciocca, S., Angelico, F. and Cantafora, A., An improved gas-liquid chromatographic method for the determination of fecal neutral sterols, J Lipid Res, 1983, 24: 332-335.

31 Setchell, K. D., Lawson, A. M., Tanida, N. and Sjovall, J., General methods for the analysis of metabolic profiles of bile acids and related compounds in feces, J Lipid Res, 1983, 24: 1085-1100.
32 Plosch, T., Kok, T., Bloks, V. W., Smit, M. J., Havinga, R., Chimini, G., Groen, A. K. and Kuipers, F., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1, J Biol Chem, 2002, 277: 33870-33877.
33 Mashige, F., Imai, K. and Osuga, T., A simple and sensitive assay of total serum bile acids, Clin Chim Acta, 1976, 70: 79-86.
34 Kuipers, F., Havinga, R., Bosschieter, H., Toorop, G. P., Hindriks, F. R. and Vonk, R. J., Enterohepatic circulation in the rat, Gastroenterology, 1985, 88: 403-411.
35 Windler, E., Zyriax, B. C., Kuipers, F., Linseisen, J. and Boeing, H., Association of plasma phytosterol concentrations with incident coronary heart disease Data from the CORA study, a case-control study of coronary artery disease in women, Atherosclerosis, 2009, 203: 284-290.
36 Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D. and Williams, R. A., Quantitative assessment of atherosclerotic lesions in mice, Atherosclerosis, 1987, 68: 231-240.
37 Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 1959, 37: 911-917.
38 Böttcher, C. S. F., van Gent, C. M. and Fries, C., A rapid and sensitive sub-micro phosphorus determination., Anal Chim Acta, 1961, 24: 203-204.
39 Stricker, D., BrightStat.com: free statistics online, Comput Methods Programs Biomed, 2008, 92: 135-143.
40 Caligiuri, G., Levy, B., Pernow, J., Thoren, P. and Hansson, G. K., Myocardial infarction mediated by endothelin receptor signaling in hypercholesterolemic mice, Proc Natl Acad Sci U S A, 1999, 96: 6920-6924.
41 Wikstrom, J., Gronros, J., Bergstrom, G. and Gan, L. M., Functional and morphologic imaging of coronary atherosclerosis in living mice using high-resolution color Doppler echocardiography and ultrasound biomicroscopy, J Am Coll Cardiol, 2005, 46: 720-727.
42 Slattery, M. L. and Jacobs, D. R., Jr., Physical fitness and cardiovascular disease mortality. The US Railroad Study, Am J Epidemiol, 1988, 127: 571-580.
43 Sesso, H. D., Paffenbarger, R. S., Jr. and Lee, I. M., Physical activity and coronary heart disease in men: The Harvard Alumni Health Study, Circulation, 2000, 102: 975-980.
44 Sharp, J. D. and Freeman, C. B., Patterns and Predictors of Uptake and Adherence to Cardiac Rehabilitation, Journal of Cardiopulmonary Rehabilitation & Prevention July/August, 2009, 29: 241-247.
45 Kamiya, A., Ohsawa, I., Fujii, T., Nagai, M., Yamanouchi, K., Oshida, Y. and Sato, Y., A clinical survey on the compliance of exercise therapy for diabetic outpatients, Diabetes Research and Clinical Practice, 1995, 27: 141-145.
46 Stypmann, J., Engelen, M. A., Epping, C., van Rijen, H. V., Milberg, P., Bruch, C., Breithardt, G., Tiemann, K. and Eckardt, L., Age and gender related reference values for transthoracic Doppler-echocardiography in the anesthetized CD1 mouse, Int J Cardiovasc Imaging, 2006, 22: 353-362.
47 Kuipers, I., Li, J., Vreeswijk-Baudoin, I., Koster, J., van der Harst, P., Sillje, H. H., Kuipers, F., van Veldhuisen, D. J., van Gilst, W. H. and de Boer, R. A., Activation of liver X receptors with T0901317 attenuates cardiac hypertrophy in vivo, Eur J Heart Fail.
48 Weiss, R. M., Ohashi, M., Miller, J. D., Young, S. G. and Heistad, D. D., Calcific aortic valve stenosis in old hypercholesterolemic mice, Circulation, 2006, 114: 2065-2069.
49 Garcia, J. A., dos Santos, L., Moura, A. L., Ricardo, K. F., Wanschel, A. C., Shishido, S. M., Spadari-Bratfisch, R. C., de Souza, H. P. and Krieger, M. H., S-nitroso-N-acetylcysteine (SNAC) prevents myocardial alterations in hypercholesterolemic LDL receptor knockout mice by antiinflammatory action, J Cardiovasc Pharmacol, 2008, 51: 78-85.

50 Herrema, H., Meissner, M., van Dijk, T. H., Brufau, G., Boverhof, R., Oosterveer, M. H., Reijngoud, D. J., Muller, M., Stellaard, F., Groen, A. K. and Kuipers, F., Bile salt sequestration induces hepatic de novo lipogenesis through farnesoid X receptor- and liver X receptor alpha-controlled metabolic pathways in mice, Hepatology, 51: 806-816.
51 Reihner, E., Angelin, B., Bjorkhem, I. and Einarsson, K., Hepatic cholesterol metabolism in cholesterol gallstone disease, J Lipid Res, 1991, 32: 469-475.
52 Shepherd, J., Packard, C. J., Bicker, S., Lawrie, T. D. and Morgan, H. G., Cholestyramine promotes receptor-mediated low-density-lipoprotein catabolism, N Engl J Med, 1980, 302: 1219-1222.
53 Carrella, M., Ericsson, S., Del Piano, C., Angelin, B. and Einarsson, K., Effect of cholestyramine treatment on biliary lipid secretion rates in normolipidaemic men, J Intern Med, 1991, 229: 241-246.
54 Davidson, M. H., Dillon, M. A., Gordon, B., Jones, P., Samuels, J., Weiss, S., Isaacsohn, J., Toth, P. and Burke, S. K., Colesevelam hydrochloride (cholestagel): a new, potent bile acid sequestrant associated with a low incidence of gastrointestinal side effects, Arch Intern Med, 1999, 159: 1893-1900.
55 Bays, H. and Dujovne, C., Colesevelam HCl: a non-systemic lipid-altering drug, Expert Opin Pharmacother, 2003, 4: 779-790.

CHAPTER 8

General Discussion

GENERAL DISUCSSION

Lifestyle in western society has become increasingly sedentary while at the same time people are exposed to relatively cheap, more energy dense, low quality foods and experience more chronic stress. As a result, imbalances in various functional aspects of life are emerging and the prevalence of conditions impairing physiological and mental functioning has risen dramatically.

Described clinically by the presence of several metabolic impairments [1], the Metabolic Syndrome (MetS) has emerged as a major health concern in western society. Because of the increasing prevalence and economic burden of MetS, efficient strategies targeting its complications are warranted.

Type 2 diabetes is the most common complication of MetS. Type 2 diabetes accelerates atherosclerosis, the major cause of death in the world. To a large degree, type 2 diabetes and atherosclerosis can be attributed to imbalances in glucose and cholesterol metabolism. Thus, restoring balance in glucose and cholesterol metabolism comprises a central feature within the treatment strategies for the MetS. Physical activity and bile acid sequestrants (BAS), the former as lifestyle intervention and the latter as pharmacological intervention, act beneficial on glucose and cholesterol homeostasis under various metabolic imbalances implicated in MetS [2-21]. However, the mediators underlying the benefits of physical activity and BAS on MetS risk factors are unclear.

The aim of this thesis was to evaluate whether the physical activity and BAS-induced improvements in MetS risk factors are underlying specific modulations in glucose and cholesterol metabolism. In the studies of this thesis we utilized Colesevelam HCL as BAS treatment and voluntary wheel running as physical activity intervention. Colesevelam HCL is a new generation BAS which displays more efficiency and an improved side effect profile compared to conventional BAS [2, 22] . Voluntary wheel running is a low stress intervention, that contrary to forced exercise strategies does not change the eating behavior of mice [23, 24].

We evaluated the effect of BAS-treatment on glycemic control and atherosclerosis. We further established that physical activity beneficially modulates cholesterol

metabolism in lean mice and subsequently applied these findings within an atherosclerotic mouse model exposed to a running wheel. Furthermore, we tested whether a combination treatment of BAS with physical activity would yield further improvements in cholesterol homeostasis and atherosclerosis reduction compared to BAS treatment by itself.

Bile acid sequestration in the Metabolic Syndrome

Utilizing engineered mouse models that display specific metabolic derangements, we show in this thesis that Colesevelam HCL, as a specifically engineered BAS, is effective in improving a variety of Mets risk factors and associated complications. We first established that BAS induced improvements in plasma glucose levels (Chapter 2 and 3), HOMA-index and insulin sensitivity in the leptin- receptor deficient obese and diabetic *db/db* mouse (Chapter 3), which parallels the BAS-induced improvements in glucose homeostasis reported for type 2 diabetic humans and other animal models of diabetes [25-28]. Then, confirming clinical findings in hypercholesterolemic patients [4, 5, 7, 29-31], we established that BAS provoked profound improvements in plasma lipids and lipoprotein profiles in atherogenic hypercholesterolemic mice (Chapter 7) and showed for the first time that BAS, substantially decreased atherosclerotic lesion size development in mice by its profound effects on cholesterol homeostasis (Chapter 7).

Bile acid sequestration and glucose homeostasis

BAS have recently emerged as agents that lower blood glucose and HbA1c levels in type 2 diabetes [14]. So far, however the actual changes in hepatic and / or peripheral glucose metabolism upon BAS have remained largely elusive. We hypothesized that BAS improve glycemic control by depleting the bile acid pool, thereby altering the effects of various nuclear receptors that have implications for bile acid, lipid and glucose homeostasis. To test this we carried out two studies using stable isotope techniques for analysis of glucose, cholesterol and bile acid metabolism in healthy lean and obese diabetic *db/db* mice fed normal chow supplemented with Colesevelam HCL (BAS) for two weeks. In one study (Chapter 2) we established the actual effects of BAS on bile acid fluxes and lipid metabolism.

In another study we (Chapter 3), we tested whether the BAS-induced improvements in glucose homeostasis are brought about by modulations in hepatic glucose fluxes.

Measuring the bile acid kinetics of cholate (Chapter 2), we first showed that *db/db* mice display an altered bile acid metabolism and thereby support the previously reported increase in the bile acid pool size in *db/db* mice [32] . Additionally, we, for the first time, showed an increased rate of bile acid synthesis in *db/db* mice. Surprisingly, BAS had no effect on the bile acid pool size nor biliary bile acid secretion rates despite a massively induced bile acid malabsorption in lean and *db/db* mice (Chapter 2). A lack of a BAS effect on bile acid pool size was recently confirmed in healthy and type 2 diabetic humans [33]. Moreover, the BAS-induced bile acid malabsorption and subsequent increased turnover of cholesterol (as demonstrated by increased *de novo* cholesterol and bile acid biosynthesis) coincided with an increase in hepatic lipogenesis that was dependent on FXR and LXR. FXR and LXR have established roles in various metabolic pathways including bile acid and lipid metabolism. The BAS-induced increase in lipogenesis can potentially be attributed to reduced FXR activation. Activated by bile acids in the liver, FXR acts to inhibit lipogenesis by inhibiting LXR-induced activation of SREBP1c [34], which is a lipogenic transcription factor. Furthermore, BAS increased *de novo* cholesterol synthesis, which was expected in order to generate substrate for bile acid synthesis. Intriguingly, the BAS-induced increased turnover of cholesterol did not reduce cellular cholesterol stores, in fact hepatic cholesterol content tended to be increased, reflecting that the increase in cholesterol synthesis was massive and more than to generate substrates for the compensatory increase in *de novo* bile acid synthesis. Such massive increase in cholesterol biosynthesis could allow for generation of oxysterols which activate LXR, and thereby lipogenesis [35].

Further, we, and others [36-38], thought that BAS mediate their glucose lowering actions through direct interference of bile acid signaling pathways in the enterohepatic system, because those pathways also involve nuclear receptors with established roles in glucose homeostasis, specifically LXR and FXR [38-41]. Modulation of such pathways could then translate into altered hepatic glucose

metabolism and thus improve glucose homeostasis. Herein, we suspected the bile acid pool size to decrease upon BAS leading to decreased signaling via FXR.. As discussed above, BAS treatment did not alter the bile acid pool size in *db/db* mice and as discussed below, the BAS-induced improvements in glucose homeostasis were not mediated by BAS effects on hepatic glucose metabolism. This implies that BAS improve glucose homeostasis by alternate pathways not primarily linked to nuclear receptors implicated in bile acid and glucose homeostasis.

By measuring *in vivo* glucose metabolism using the MIDA approach, we found that BAS treatment did not bring about its glucose lowering actions by improving the specific disturbances in hepatic glucose metabolism that we observed for *db/db* mice (Chapter 3). Compared to lean mice, *db/db* mice displayed an increased glucokinase flux, glucose cycling rate and total hepatic glucose production rate. Although treatment with BAS decreased blood glucose levels and *de novo*-glucose-6-phosphate synthesis in *db/db* mice, BAS treatment did not lead to ameliorations in any of these above stated disturbances of hepatic glucose metabolism. Therefore, the liver could not be the mediator of the glucose lowering actions of BAS. However, we observed that BAS targeted peripheral tissues. Intriguingly, the BAS-induced increase in glucose metabolic clearance rate in *db/db* mice coincided with a reduced long-chain acylcarnitine content in their skeletal muscles. Long-chain acylcarnitines are intermediates of ß-oxidation. High intracellular levels of these molecules can be toxic and have shown to be reflective of an overloaded fatty acid oxidation. The consequence of this lack of capacity is an accumulation of substrates such as acyl-CoA's in the mitochondria [42], and ceramide and diacylglycerides in the cytosol, which in turn interfere with insulin stimulated glucose oxidation [42, 43]. Therefore, a BAS-induced reduction in skeletal muscle long-chain acylcarnitine content is reflective of a more efficient mitochondrial fatty acid oxidation. Concomitantly to the BAS-induced decreases in plasma fatty acid levels, BAS treated *db/*db mice displayed an improved HOMA-index and increased glucose metabolic clearance rate, alltogether indicative of an improved insulin sensitivity. Future studies elucidating the effects of BAS treatment on the skeletal muscle insulin signaling cascade as well as on

mitochondrial respiration are desirable in further determination of the specific effects of BAS in improving peripheral insulin sensitivity.

The question is how BAS, by altering the enterohepatic circulation of bile acids, promote an improved insulin sensitivity in peripheral tissue. Which might be the responsible factors herein? Known for their effects on increasing metabolic rate, thyroid hormones might have a potential role. TGR5 activation in brown adipose tissue and skeletal muscle stimulates the conversion of inactive to active thyroid hormone which then by specific signaling pathways increases metabolic rate [44]. However, the conversion of inactive to active thyroid hormone is catalyzed through activation of TGR5 by bile acids. Thus, granted that plasma bile acid levels were lower in BAS treated mice (Chapter 3) an implication for peripheral TGR5 activation on BAS-induced glycemic control seems rather unlikely. Another candidate for the BAS-induced peripheral actions on glucose homeostasis is an incretin called glucagon-like-peptide-1 (GLP-1). BAS have recently been shown to increase the production of GLP-1 levels in insulin resistant, diet-induced obese rats and this has been associated with improved insulin sensitivity [11]. Moreover, incretins have been proven effective in treating type 2 diabetes [45]. Intriguingly, we observed an increased GLP-1 expression in the ileum of BAS treated *db/db* mice (Chapter 3, data not shown). Concomitantly, BAS induced a slight malabsorption of dietary fatty acids in lean and *db/db* mice (Chapter 3, data not shown). This suggests that upon BAS treatment an increased amount of fatty acids is passing through the distal parts of the intestine, where fatty acids have been shown to induce GLP-1 by stimulation of L-cells [46].

From our data and within the limits of our methods we concluded that changes in liver glucose metabolism do not mediate the glucose-lowering effect of BAS. Utilizing the MIDA approach, however, we were not able to measure glycolysis. Thus, we cannot exclude a possibility for BAS to enhance glycolysis and to thereby affect hepatic and whole-body glucose metabolism. Indeed, upon BAS-treatment there was a trend for an increased glucokinase flux and an induction of pyruvate kinase, a glycolytic gene, in *db/db* mice. These observations in combination with the increased hepatic triglyceride contents in BAS treated mice (Chapter 2 and 3) which appeared to be mediated by an increased *de novo*

synthesis of fatty acids and fatty acid chain elongation (Chapter 2) are, in fact, speculative of an increased flux of glucose through glycolysis. Thus, despite the fact that BAS treatment does not decrease hepatic glucose output, BAS potentially mediated an increased flux of glucose into hepatic triglyceride storage, further study is warranted to substantiate this hypothesis. However, given the substantial improvements in peripheral glucose homeostasis upon BAS treatment, it seems rather unlikely that possible BAS-mediated increases in hepatic glycolysis have a major contribution to the improved glycemic control.

Bile acid sequestration and cholesterol homeostasis

Before statins emerged as the mainstream pharmacological treatment for hypercholesterolemic patients, BAS were the first choice to treat hypercholesterolemia. Numerous clinical studies demonstrated BAS to substantially improve total and LDL-cholesterol levels which was accompanied by considerable relative risk reductions in fatal and nonfatal myocardial infarction [29], as well as increased regression [4, 6, 7] and reduced progression [4-7] in coronary artery lesions. However, during their clinical use in the 1980s BAS treatment, then limited to cholestyramine and colestipol, proofed difficult due to poor tolerability pertaining to gastrointestinal side effects and due to their interactions with other commonly prescribed drugs [22]. In the 1990s, Colesevelam HCL was specifically engineered for much better tolerability, efficacy and much reduced potential for drug interactions [2]. While the cholesterol lowering effects of Colesevelam HCL have been widely reported [3, 8, 9, 21] and it even has been the first drug to be FDA-approved for its effects on improving both glycemic control and cholesterol levels, its effects on atherosclerosis have not been reported so far.

We evaluated the effects of Colesevelam HCL (BAS) on atherosclerosis in the Ldlr-deficient mouse (Chapter 7), an established mouse model of atherosclerosis. We observed profound effects of BAS on cholesterol homeostasis. BAS provoked a considerable switch from cholesterol accumulation to cholesterol elimination which was accompanied by a substantial reduction in atherosclerotic lesion size development, by ~78%. Clearly, our data warrant the re-evaluation of BAS using Colesevelam HCL on atherosclerotic lesion size reduction (and

progression) in human trials. The effect of Colesevalam in mice not only highlights the central role of cholesterol in the pathogenesis of atherosclerosis but also shows that atherosclerosis development can be effectively reduced when targeting cholesterol accumulation. Intriguingly, fecal sterol excretion was increased but biliary sterol secretion was markedly reduced upon BAS treatment, indicating that a non-hepatobiliary cholesterol excretion pathway accounted for the negative cholesterol balance upon BAS. Upon calculation, we indeed found such pathways to be enhanced at least 4-fold by BAS treatment.

The nature of this non-hepatobiliary cholesterol excretion pathway needs to be determined in future experiments. However, the existence of a transintestinal cholesterol efflux (TICE) route has over the last years gained considerable appreciation as more evidence has been gathered showing the intestine to actively secrete cholesterol [47]. Our data is suggestive of an enhanced TICE under BAS treatment. From our calculations, however, we cannot conclude which bodily cholesterol pools contributed to the BAS-induced increase in cholesterol excretion. Unfortunately, we were not able to quantify cholesterol absorption in these experiments. The low plasma plant sterol / cholesterol ratio, a surrogate marker of cholesterol absorption, is a first suggestion that BAS reduced cholesterol absorption. BAS could provoke a reduced cholesterol absorption by interfering with micelle formation. For absorption cholesterol needs to be incorporated into mixed micelles, since bile acids are bound to BAS, mixed micelle formation is most likely impaired. Future studies analyzing *in vivo* cholesterol fluxes, directly measuring cholesterol absorption, synthesis and contribution of intestinal shedding will be necessary to quantify cholesterol balance in these experiments.

2. Physical activity in the Metabolic Syndrome

Utilizing voluntary wheel running as a physical activity intervention to target MetS risk factors and associated complications, we observed improvements in a variety of MetS risk factors throughout the studies in this thesis. For example, physical activity led to a reduced bodyweight gain, reduced epididymidal white adipose tissue weight, improved lipoprotein profiles in hypercholesterolemic atherosclerotic mice (Chapter 5) and improved plasma lipid values in healthy lean chow-fed mice

(Chapter 4) and hypercholesterolemic atherosclerotic mice (Chapter 5). Thereby we confirm a long known beneficial role for physical activity in improving MetS risk factors like obesity [48-50] and hyperlipidemia [50, 51]. Furthermore, we show that a sufficient amount of physical activity significantly reduces atherosclerosis development in hypercholesterolemic atherosclerotic mice (Chapter 5), while a decreased amount is not clearly effective (Chapter 6). Thus, we not only confirm clinical and experimental studies reporting beneficial effects of physical activity on atherosclerosis [15-20, 52, 53] but also that a higher level of physical activity leads to greater effects on cardiovascular risk reduction [13, 54].

Despite the well established role of physical activity on MetS risk factors and complications, the mechanisms underlying these effects remain elusive. Inspired by limited past work showing that physical activity provokes alterations in cholesterol and bile acid metabolism [55-58], we questioned whether physical activity may enhance pathways leading to enhanced fecal sterol excretion (Chapter 4 and 5) and if so, whether these pathways may play a considerable role in the physical activity-induced reduction of MetS risk factors and its major complication atherosclerosis (Chapter 6 and 7).

Physical activity and cholesterol metabolism

Instead of controlling the intensity of physical activity by exposing mice to forced exercise training, we, for a number of reasons, choose for the voluntary wheel running set up. First, it has previously been shown that already a two week exposure of healthy mice to a voluntary running wheel results in cardiac and skeletal muscle adaptations that are consistent with those of endurance exercise [59]. Second, the voluntary wheel running set up provides an anti-stress intervention [23] compared to forced exercise interventions, which have been found to cause chronic stress-like changes in the hypothalamic-pituitary-adrenal axis [24]. Third, reflecting the stress-response, upon forced exercise rodents do not increase their food intake [60], while rodents engaged in voluntary activity increase their food intake to compensate for the increased energy expenditure. Thus, the stress associated with forced exercise regimes results in an energy imbalance which by itself could alter parameters of cholesterol turnover. As shown in chapter 4 we observed

indeed that physical activity as in voluntary wheel running enhanced whole body cholesterol turnover by decreasing cholesterol absorption and stimulating bile acid synthesis in healthy chow fed mice. In hypercholesterolemic, atherosclerotic mice, we then showed that the reduction in atherosclerotic lesion size development coincided with an enhanced cholesterol turnover especially mediated by increased cholesterol turnover into bile acids, while cholesterol absorption did not appear affected in this model (Chapter 6). The ~45% reduction in atherosclerotic lesion size development that we found upon voluntary exercise here, parallels previous studies that observed reductions ranging from 30-54% upon forced exercise in atherosclerotic hypercholesterolemic mouse models [10, 12, 18, 19]. No study, however, has linked the beneficial effects of physical activity observed on atherosclerosis reduction to an increased cholesterol turnover before. Thus, by demonstrating here, for the first time, that physical activity increases cholesterol turnover while concomitantly reducing atherosclerotic lesion size development, we indicate that an increased cholesterol turnover might be a considerable driving factor for the physical activity-induced atherosclerosis reduction. .

Intriguingly, the enhanced cholesterol turnover upon physical activity was specific for an increased cholesterol conversion into bile acids. While physical activity induced marked increases in biliary bile acid secretions (up to 68%) and fecal bile acid loss (up to 93%), biliary secretion rates of cholesterol were unaffected and fecal excretion of neutral sterols was increased to a lesser degree (up to 34%) than the excretion of bile acids. The increased fecal bile acid excretion in physically active mice reflects an increase in *de novo* bile acid synthesis. To our surprise, the transcriptional machinery implicated in bile acid synthesis was not affected by exercise. Studying *in vivo* bile acid kinetics would provide detailed information on the actual alterations of physical activity on different aspects of bile acid metabolism and would be desirable. However, increases in *de novo* bile acid synthesis without concomitant increases in any of the major bile acid genes have also been reported by others [61, 62] and most likely underlie posttranscriptional mechanisms. It is speculative therefore that physical activity regulates bile acid synthesis via metabolic control mechanisms. The increase in bile acid synthesis may be a physiological response on the increased energy demand. We observed

that physical activity increased dietary fatty acid absorption (Chapter 6, data not shown). During physical activity, energy is depleted in energy expending tissues, such as skeletal muscle. Thus, a high demand for new energy manifests in these tissues. In this regard, physical activity might, hypothetically, increase bile acid synthesis to increase the capacity for micelle formation; thereby fatty acid absorption and thus energy delivery to energy expending tissues.

Importantly, although cholesterol turnover was found increased upon voluntary wheel running in healthy chow-fed mice as demonstrated by various parameters (Chapter 4), we did not observe an increased *in vivo* macrophage-to-feces RCT in healthy chow-fed mice exposed the same experimental set-up (Chapter 5). To our surprise, despite unchanged HDL levels and *in vivo* RCT, running mice displayed an increased cholesterol efflux from macrophages towards plasma (Chapter 5). An increased efflux capacity in trained athletes has previously been reported, however, with concomitantly higher levels in HDL [63]. From human data it is known, that trained endurance athletes have substantially higher HDL levels than healthy sedentary controls [64, 65]. Moreover, it is known that physical activity increases HDL levels in previously sedentary individuals very modestly [65, 66], thus not substantially affecting the difference in HDL levels to trained endurance athletes [65]. To make the issue even more complex, it has also been reported that runners have a ~2-time increased biological half-life of HDL proteins and that runners do not produce more HDL protein but catabolize less [64]. However, no previous study has reported the effects of physical activity on HDL levels in healthy chow-fed mice. Moreover, differences in lipoprotein metabolism between mice and men have been documented, some of them directly pertaining to HDL metabolism[67-69], likely explaining the lack of effect of physical activity on HDL levels we observed. Finally, HDL by itself may not be the best indicator for RCT *per se*, recent reports demonstrate it to be a rather dynamic cholesterol transporter with varying function and structure [70, 71]. It has been suggested that preβ_1-HDL might be a better marker for RCT than HDL [72], however, physical activity has shown differential effects on preβ_1-HDL levels [63, 66].

The puzzling finding then remains, why *in vivo* macrophage-to-feces RCT was unaltered despite increased cholesterol turnover, as shown by an enhanced

sterol excretion, and an increased cholesterol efflux from macrophages towards plasma in two-week running healthy chow-fed mice? The effects of voluntary wheel running on parameters of cholesterol metabolism are rather small (Chapter 4), and the utilized method may possibly not be sensitive enough to pick up subtle changes. In fact, tracer recovery in livers and feces (bile acid and neutral sterol fractions) showed subtle, albeit not significant, increases in running mice (Chapter 5). It is desirable to test *in vivo* macrophage-to-feces RCT in hypercholesterolemic, atherosclerotic mice running for 12 weeks, where effects of running were more striking compared to healthy chow-fed mice, especially pertaining to biliary bile acid secretion and fecal bile acid loss as already stated above. More work is needed to unravel the enigma of *in vivo* macrophage-to-feces RCT upon exercise.

Beneficial effects of physical activity on hepatic triglyceride content

Utilizing the voluntary running wheel as a lifestyle intervention for studies described in this thesis, we consistently observed reductions in hepatic fat deposition (Chapters 4 to 7). Increased hepatic fat deposition is associated with MetS, type 2 diabetes and cardiovascular disease [73], thus the favorable effect of voluntary wheel running on reducing hepatic fat storage provides further benefit in MetS risk reduction and is discussed below.

Sources for hepatic triglycerides are free fatty acids from adipose tissue, *de novo* lipogenesis and chylomicron-derived fatty acids from dietary fat. In addition, hepatic triglyceride concentration is a function of hepatic oxidation and clearance of VLDL. Both processes might be favorable altered upon exercise but thorough studies are missing to date. There is a paucity of data showing the effects of physical activity on fatty liver in humans. An array of studies, however, shows that physical activity combined with dietary intervention yields improvements in liver tests and ameliorates fatty liver and weight loss has been found fundamental in a reduction of fatty liver in humans [74]. Recently, however, cardiorespiratory fitness was found to be the best predictor of changes in hepatic triglycerides independent of total and visceral adiposity [75].

It is intriguing that lean and hypercholesterolemic mice exposed to a running wheel display reduced hepatic triglyceride stores, the magnitude of

reduction compared to respective control mice ranging from 1.4 to 1.9-fold (Chapters 4 to 7). This reduction was accompanied by reduced expression of key lipogenic genes (SREBP1c, FASN and SCD1) in running hypercholesterolemic atherosclerotic mice (Chapter 6). Comparing hepatic triglycerides contents across the studies described in this thesis, it becomes apparent that Ldlr-deficient mice fed a western-type diet display enhanced triglyceride storage (Chapter 6 and 7) compared to chow-fed wildtype mice (Chapter 4 and 5). To date there are no reports describing the effects of physical activity, either forced or voluntary, on hepatic lipids in hypercholesterolemic mouse models. Yet, limited data show that high fat and low fat-fed wildtype mice display reduced hepatic triglyceride stores after treadmill exercise training [76, 77] and that swim training reduced hepatic fatty acid synthesis in C57BL6/J mice [78].

The exact mediators pertaining to the beneficial effects of regular physical activity on hepatic triglycerides remain, so far, elusive and is beyond the scope of this thesis. However, regular physical activity promotes specific skeletal muscle adaptations (i.e.: increased mitochondrial biogenesis, capillarization) that allow for an increased uptake of fatty acids, β-oxidation and skeletal muscle triglyceride storage [79]. These are all adaptations that become necessary to sustain a delivery of nutrients to this tissue during physical activity, and that may therefore also serve to partition fatty acids away from the liver.

Effects of physical activity on cholesterol metabolism are dose-dependent

We observed considerable differences in magnitude of running wheel activity and outcome parameters between two groups of hypercholesterolemic atherosclerotic mice of two different studies (Chapter 6 and 7). Although the experimental set-up was identical, the mice in the experiment of chapter 5 ran twice as much compared to the mice used in the experiment of chapter 6. Interestingly, the “lazy” mice showed no decrease in plasma cholesterol and diminished improvement of plaque formation indicating a direct relation between the extend of physical activity and these parameters. The difference in running wheel activity between the two studies closely paralleled the discrepancy in magnitude reduction of atherosclerotic lesion size between the two studies: mice

running twice as much displayed twice as much reduction in atherosclerotic lesion size development (Chapter 6 vs. Chapter 7). In fact calculating the area under the curve for the average distance ran per day for every one of the 12 weeks and plotting this against lesion size for running mice of Chapter 6 and 7, we found a significant correlation between area under the curve and lesion size depicted in Figure 1 (Spearman r= -0.63, *P*=0.044).

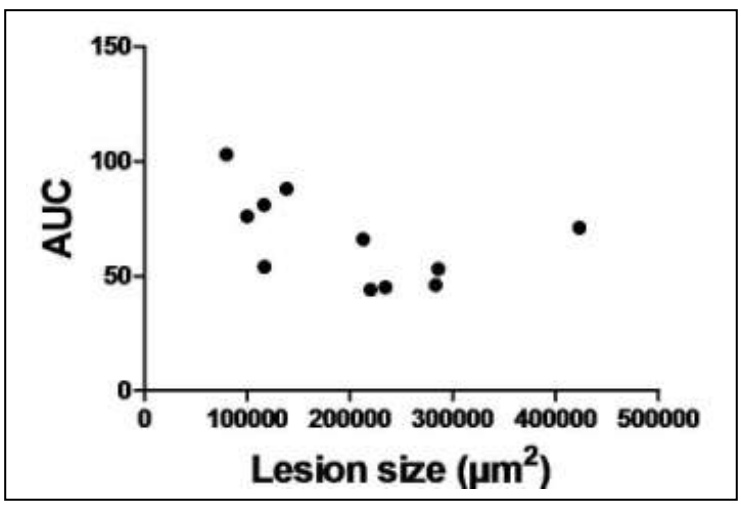


Figure 1. Atherosclerotic lesion size and running wheel activity are significantly correlated. The area under the curve (AUC) was calculated by the average distance ran per day for every 12 weeks of the running intervention. Subsequently AUC was plotted against atherosclerotic lesion size for running mice of chapter 6 and 7.

Physical activity and bile acid sequestration as co-treatments in atherosclerosis

As stated above, physical activity programs often do not result in sufficient risk reduction for reasons related to poor compliance or a more advanced risk profile of the subject. In these situations pharmacological intervention becomes necessary and is either prescribed by itself or as combination therapy with a lifestyle intervention, such as physical activity.

BAS and voluntary wheel running by themselves clearly have differential effects on cholesterol metabolism. The BAS-induced reduction in atherosclerosis coincided with a switch from a positive to a negative cholesterol balance with concomitant increases in non-hepatobiliary cholesterol excretion. In contrast, the reduction in atherosclerotic lesion size upon voluntary wheel running coincided with a marked effect on cholesterol turnover into bile acids. Thus, we questioned whether a combination of the two treatments would provide further benefits on

atherosclerosis and cholesterol metabolism. We found that such a combination treatment did not yield additional benefits to BAS treatment alone, most likely because BAS effects on sterol metabolism are more dramatic and may override the more subtle effects of voluntary wheel running.

Furthermore, our data clearly show that under severe hypercholesterolemia, as in Ldlr deficiency, BAS treatment is much more effective in the reduction of atherosclerosis than voluntary wheel running. While, depending on activity levels, we found a range of ~20 – 45% in reduction of atherosclerotic lesion size in mice exposed to a voluntary running wheel (Chapter 6 and 5, respectively), the BAS effect was clearly stronger, yielding a reduction of ~78% (Chapter 6). Similar observations were found for fecal and biliary parameters of cholesterol metabolism.

Perspectives and conclusion

The studies described in this thesis provide novel insights concerning cholesterol metabolism-modulated amelioration of MetS risk factors and associated complications by BAS and physical activity. As stated in the discussion, questions arose specifically related to the mechanisms of a BAS-induced reduction of skeletal muscle long-chain acylcarnitines, the BAS-induced enhanced non-hepatobiliary cholesterol excretion and the physical activity-stimulated bile acid synthesis and *in vivo* macrophage-to-feces RCT. Using sophisticated methods, future studies are warranted to address these questions.

Furthermore, the presented studies focused on the effect of BAS and physical activity in the development of complications associated with MetS using extreme mouse models. This thesis demonstrates that BAS and physical activity are beneficial in reducing MetS risk factors and associated complications when presenting with increased MetS risk suggesting a positive role for both treatments in managing the currently ever-increasing prevalence of MetS

It now would be desirable to evaluate both treatments in the following:

1. The prevention of MetS and its complications and test the effects of early-life treatment and treatment maintenance on development of MetS and associated complications later in life.

2. The regression of already established complications such as advanced atherosclerosis.

REFERENCES

1 Grundy, S. M., Brewer, H. B., Jr., Cleeman, J. I., Smith, S. C., Jr. and Lenfant, C., Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition, Circulation, 2004, 109: 433-438.
2 Welchol Product Information. Colesevelam Hydrochloride, In, Accessed July 8 2010.
3 Bays, H. and Jones, P. H., Colesevelam hydrochloride: reducing atherosclerotic coronary heart disease risk factors, Vasc Health Risk Manag, 2007, 3: 733-742.
4 Blankenhorn, D. H., Nessim, S. A., Johnson, R. L., Sanmarco, M. E., Azen, S. P. and Cashin-Hemphill, L., Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts, JAMA, 1987, 257: 3233-3240.
5 Brensike, J. F., Levy, R. I., Kelsey, S. F., Passamani, E. R., Richardson, J. M., Loh, I. K., Stone, N. J., Aldrich, R. F., Battaglini, J. W., Moriarty, D. J. and et al., Effects of therapy with cholestyramine on progression of coronary arteriosclerosis: results of the NHLBI Type II Coronary Intervention Study, Circulation, 1984, 69: 313-324.
6 Brown, G., Albers, J. J., Fisher, L. D., Schaefer, S. M., Lin, J. T., Kaplan, C., Zhao, X. Q., Bisson, B. D., Fitzpatrick, V. F. and Dodge, H. T., Regression of coronary artery disease as a result of intensive lipid-lowering therapy in men with high levels of apolipoprotein B, N Engl J Med, 1990, 323: 1289-1298.
7 Cashin-Hemphill, L., Mack, W. J., Pogoda, J. M., Sanmarco, M. E., Azen, S. P. and Blankenhorn, D. H., Beneficial effects of colestipol-niacin on coronary atherosclerosis. A 4-year follow-up, JAMA, 1990, 264: 3013-3017.
8 Davidson, M. H., Dillon, M. A., Gordon, B., Jones, P., Samuels, J., Weiss, S., Isaacsohn, J., Toth, P. and Burke, S. K., Colesevelam hydrochloride (cholestagel): a new, potent bile acid sequestrant associated with a low incidence of gastrointestinal side effects, Arch Intern Med, 1999, 159: 1893-1900.
9 Handelsman, Y., Abby, S. L., Jin, X., Donovan, J. M. and Jones, M. R., Colesevelam HCl improves fasting plasma glucose and lipid levels in patients with prediabetes, Postgrad Med, 2009, 121: 62-69.
10 Meilhac, O., Ramachandran, S., Chiang, K., Santanam, N. and Parthasarathy, S., Role of arterial wall antioxidant defense in beneficial effects of exercise on atherosclerosis in mice, Arterioscler Thromb Vasc Biol, 2001, 21: 1681-1688.
11 Shang, Q., Saumoy, M., Holst, J. J., Salen, G. and Xu, G., Colesevelam improves insulin resistance in a diet-induced obesity (F-DIO) rat model by increasing the release of GLP-1, Am J Physiol Gastrointest Liver Physiol, 298: G419-424.
12 Shimada, K., Kishimoto, C., Okabe, T. A., Hattori, M., Murayama, T., Yokode, M. and Kita, T., Exercise training reduces severity of atherosclerosis in apolipoprotein E knockout mice via nitric oxide, Circ J, 2007, 71: 1147-1151.
13 Slattery, M. L. and Jacobs, D. R., Jr., Physical fitness and cardiovascular disease mortality. The US Railroad Study, Am J Epidemiol, 1988, 127: 571-580.
14 Staels, B., A review of bile acid sequestrants: potential mechanism(s) for glucose-lowering effects in type 2 diabetes mellitus, Postgrad Med, 2009, 121: 25-30.
15 Ajijola, O. A., Dong, C., Herderick, E. E., Ma, Q., Goldschmidt-Clermont, P. J. and Yan, Z., Voluntary running suppresses proinflammatory cytokines and bone marrow endothelial progenitor cell levels in apolipoprotein-E-deficient mice, Antioxid Redox Signal, 2009, 11: 15-23.

16 Kramsch, D. M., Aspen, A. J., Abramowitz, B. M., Kreimendahl, T. and Hood, W. B., Jr., Reduction of coronary atherosclerosis by moderate conditioning exercise in monkeys on an atherogenic diet, N Engl J Med, 1981, 305: 1483-1489.
17 Napoli, C., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., D'Armiento, F. P., Crimi, E., Byrns, R. E., Casamassimi, A., Lanza, A., Gombos, F., Sica, V. and Ignarro, L. J., Physical training and metabolic supplementation reduce spontaneous atherosclerotic plaque rupture and prolong survival in hypercholesterolemic mice, Proc Natl Acad Sci U S A, 2006, 103: 10479-10484.
18 Napoli, C., Williams-Ignarro, S., De Nigris, F., Lerman, L. O., Rossi, L., Guarino, C., Mansueto, G., Di Tuoro, F., Pignalosa, O., De Rosa, G., Sica, V. and Ignarro, L. J., Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice, Proc Natl Acad Sci U S A, 2004, 101: 8797-8802.
19 Okabe, T. A., Kishimoto, C., Murayama, T., Yokode, M. and Kita, T., Effects of exercise on the development of atherosclerosis in apolipoprotein E-deficient mice, Exp Clin Cardiol, 2006, 11: 276-279.
20 Pellegrin, M., Miguet-Alfonsi, C., Bouzourene, K., Aubert, J. F., Deckert, V., Berthelot, A., Mazzolai, L. and Laurant, P., Long-term exercise stabilizes atherosclerotic plaque in ApoE knockout mice, Med Sci Sports Exerc, 2009, 41: 2128-2135.
21 Rosenson, R. S., Colesevelam HCl reduces LDL particle number and increases LDL size in hypercholesterolemia, Atherosclerosis, 2006, 185: 327-330.
22 Bays, H. and Dujovne, C., Colesevelam HCl: a non-systemic lipid-altering drug, Expert Opin Pharmacother, 2003, 4: 779-790.
23 Binder, E., Droste, S. K., Ohl, F. and Reul, J. M., Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice, Behav Brain Res, 2004, 155: 197-206.
24 Luger, A., Deuster, P. A., Kyle, S. B., Gallucci, W. T., Montgomery, L. C., Gold, P. W., Loriaux, D. L. and Chrousos, G. P., Acute hypothalamic-pituitary-adrenal responses to the stress of treadmill exercise. Physiologic adaptations to physical training, N Engl J Med, 1987, 316: 1309-1315.
25 Fonseca, V. A., Rosenstock, J., Wang, A. C., Truitt, K. E. and Jones, M. R., Colesevelam HCl improves glycemic control and reduces LDL cholesterol in patients with inadequately controlled type 2 diabetes on sulfonylurea-based therapy, Diabetes Care, 2008, 31: 1479-1484.
26 Goldberg, R. B., Fonseca, V. A., Truitt, K. E. and Jones, M. R., Efficacy and safety of colesevelam in patients with type 2 diabetes mellitus and inadequate glycemic control receiving insulin-based therapy, Arch Intern Med, 2008, 168: 1531-1540.
27 Kobayashi, M., Ikegami, H., Fujisawa, T., Nojima, K., Kawabata, Y., Noso, S., Babaya, N., Itoi-Babaya, M., Yamaji, K., Hiromine, Y., Shibata, M. and Ogihara, T., Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin, Diabetes, 2007, 56: 239-247.
28 Bays, H. E., Goldberg, R. B., Truitt, K. E. and Jones, M. R., Colesevelam hydrochloride therapy in patients with type 2 diabetes mellitus treated with metformin: glucose and lipid effects, Arch Intern Med, 2008, 168: 1975-1983.
29 The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease, JAMA, 1984, 251: 351-364.
30 Insull, W., Jr., Clinical utility of bile acid sequestrants in the treatment of dyslipidemia: a scientific review, South Med J, 2006, 99: 257-273.
31 Kane, J. P., Malloy, M. J., Ports, T. A., Phillips, N. R., Diehl, J. C. and Havel, R. J., Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens, JAMA, 1990, 264: 3007-3012.
32 Zhang, Y., Lee, F. Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F. J., Willson, T. M. and Edwards, P. A., Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice, Proc Natl Acad Sci U S A, 2006, 103: 1006-1011.

33 Brufau, G., Stellaard, F., Prado, K., Bloks, V. W., Jonkers, E., Boverhof, R., Kuipers, F. and Murphy, E. J., Improved glycemic control with colesevelam treatment in patients with type 2 diabetes is not directly associated with changes in bile acid metabolism, Hepatology.
34 Watanabe, M., Houten, S. M., Wang, L., Moschetta, A., Mangelsdorf, D. J., Heyman, R. A., Moore, D. D. and Auwerx, J., Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c, J Clin Invest, 2004, 113: 1408-1418.
35 Baranowski, M., Biological role of liver X receptors, J Physiol Pharmacol, 2008, 59 Suppl 7: 31-55.
36 Cariou, B., Duran-Sandoval, D., Kuipers, F. and Staels, B., Farnesoid X receptor: a new player in glucose metabolism?, Endocrinology, 2005, 146: 981-983.
37 Cariou, B. and Staels, B., FXR: a promising target for the metabolic syndrome?, Trends Pharmacol Sci, 2007, 28: 236-243.
38 Laffitte, B. A., Chao, L. C., Li, J., Walczak, R., Hummasti, S., Joseph, S. B., Castrillo, A., Wilpitz, D. C., Mangelsdorf, D. J., Collins, J. L., Saez, E. and Tontonoz, P., Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue, Proc Natl Acad Sci U S A, 2003, 100: 5419-5424.
39 Steffensen, K. R. and Gustafsson, J. A., Putative metabolic effects of the liver X receptor (LXR), Diabetes, 2004, 53 Suppl 1: S36-42.
40 Stulnig, T. M., Oppermann, U., Steffensen, K. R., Schuster, G. U. and Gustafsson, J. A., Liver X receptors downregulate 11beta-hydroxysteroid dehydrogenase type 1 expression and activity, Diabetes, 2002, 51: 2426-2433.
41 Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L. and Brown, M. S., Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR, Proc Natl Acad Sci U S A, 2001, 98: 6027-6032.
42 Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D. and Muoio, D. M., Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance, Cell Metab, 2008, 7: 45-56.
43 Koves, T. R., Li, P., An, J., Akimoto, T., Slentz, D., Ilkayeva, O., Dohm, G. L., Yan, Z., Newgard, C. B. and Muoio, D. M., Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency, J Biol Chem, 2005, 280: 33588-33598.
44 Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., Messaddeq, N., Harney, J. W., Ezaki, O., Kodama, T., Schoonjans, K., Bianco, A. C. and Auwerx, J., Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation, Nature, 2006, 439: 484-489.
45 Drucker, D. J. and Nauck, M. A., The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes, Lancet, 2006, 368: 1696-1705.
46 Rocca, A. S., LaGreca, J., Kalitsky, J. and Brubaker, P. L., Monounsaturated fatty acid diets improve glycemic tolerance through increased secretion of glucagon-like peptide-1, Endocrinology, 2001, 142: 1148-1155.
47 van der Velde, A. E., Brufau, G. and Groen, A. K., Transintestinal cholesterol efflux, Curr Opin Lipidol, 21: 167-171.
48 Ross, R., Dagnone, D., Jones, P. J., Smith, H., Paddags, A., Hudson, R. and Janssen, I., Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men. A randomized, controlled trial, Ann Intern Med, 2000, 133: 92-103.

49 Rice, B., Janssen, I., Hudson, R. and Ross, R., Effects of aerobic or resistance exercise and/or diet on glucose tolerance and plasma insulin levels in obese men, Diabetes Care, 1999, 22: 684-691.
50 U.S. Department of Health and Human Services CfDCaP, N. C. f. C. D. P. a. H. P., Physical Activity and Health: A Report of the Surgeon General, In, 1996.
51 Haskell, W. L., The influence of exercise on the concentrations of triglyceride and cholesterol in human plasma, Exerc Sport Sci Rev, 1984, 12: 205-244.
52 Pellegrin, M., Alonso, F., Aubert, J. F., Bouzourene, K., Braunersreuther, V., Mach, F., Haefliger, J. A., Hayoz, D., Berthelot, A., Nussberger, J., Laurant, P. and Mazzolai, L., Swimming prevents vulnerable atherosclerotic plaque development in hypertensive 2-kidney, 1-clip mice by modulating angiotensin II type 1 receptor expression independently from hemodynamic changes, Hypertension, 2009, 53: 782-789.
53 Blumenthal, J. A., Emery, C. F., Madden, D. J., George, L. K., Coleman, R. E., Riddle, M. W., McKee, D. C., Reasoner, J. and Williams, R. S., Cardiovascular and behavioral effects of aerobic exercise training in healthy older men and women, J Gerontol, 1989, 44: M147-157.
54 Sesso, H. D., Paffenbarger, R. S., Jr. and Lee, I. M., Physical activity and coronary heart disease in men: The Harvard Alumni Health Study, Circulation, 2000, 102: 975-980.
55 Sutherland, W. H., Nye, E. R., Macfarlane, D. J., Williamson, S. A. and Robertson, M. C., Cholesterol metabolism in distance runners, Clin Physiol, 1992, 12: 29-37.
56 Wilund, K. R., Feeney, L. A., Tomayko, E. J., Chung, H. R. and Kim, K., Endurance exercise training reduces gallstone development in mice, J Appl Physiol, 2008, 104: 761-765.
57 Yiamouyiannis, C. A., Martin, B. J. and Watkins, J. B., 3rd, Chronic physical activity alters hepatobiliary excretory function in rats, J Pharmacol Exp Ther, 1993, 265: 321-327.
58 Watkins, J. B., 3rd, Crawford, S. T. and Sanders, R. A., Chronic voluntary exercise may alter hepatobiliary clearance of endogenous and exogenous chemicals in rats, Drug Metab Dispos, 1994, 22: 537-543.
59 Allen, D. L., Harrison, B. C., Maass, A., Bell, M. L., Byrnes, W. C. and Leinwand, L. A., Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse, J Appl Physiol, 2001, 90: 1900-1908.
60 Dohn, G. L., Bechner, G. R., Stephenson, T. P. and Womack, M., G.L. Dohn, G.R. Bechner, T.P. Stephenson and M. Womac, Journal of Applied Physiology, 1977, 42: 753–757.
61 Hulzebos, C. V., Wolters, H., Plosch, T., Kramer, W., Stengelin, S., Stellaard, F., Sauer, P. J., Verkade, H. J. and Kuipers, F., Cyclosporin a and enterohepatic circulation of bile salts in rats: decreased cholate synthesis but increased intestinal reabsorption, J Pharmacol Exp Ther, 2003, 304: 356-363.
62 Liu, Y., Havinga, R., FR, V. D. L., Boverhof, R., Sauer, P. J., Kuipers, F. and Stellaard, F., Dexamethasone exposure of neonatal rats modulates biliary lipid secretion and hepatic expression of genes controlling bile acid metabolism in adulthood without interfering with primary bile acid kinetics, Pediatr Res, 2008, 63: 375-381.
63 Olchawa, B., Kingwell, B. A., Hoang, A., Schneider, L., Miyazaki, O., Nestel, P. and Sviridov, D., Physical fitness and reverse cholesterol transport, Arterioscler Thromb Vasc Biol, 2004, 24: 1087-1091.
64 Herbert, P. N., Bernier, D. N., Cullinane, E. M., Edelstein, L., Kantor, M. A. and Thompson, P. D., High-density lipoprotein metabolism in runners and sedentary men, JAMA, 1984, 252: 1034-1037.
65 Thompson, P., Cullinane, E., Sady, S., Flynn, M., Bernier, D., Kantor, M., Saritelli, A. and Herbert, P., Modest changes in high-density lipoprotein concentration and metabolism with prolonged exercise training, Circulation, 1988, 78: 25-34.
66 Leaf, D. A., The effect of physical exercise on reverse cholesterol transport, Metabolism, 2003, 52: 950-957.

67 Applebaum-Bowden, D., Kobayashi, J., Kashyap, V. S., Brown, D. R., Berard, A., Meyn, S., Parrott, C., Maeda, N., Shamburek, R., Brewer, H. B., Jr. and Santamarina-Fojo, S., Hepatic lipase gene therapy in hepatic lipase-deficient mice. Adenovirus-mediated replacement of a lipolytic enzyme to the vascular endothelium, J Clin Invest, 1996, 97: 799-805.
68 Lagrost, L., Regulation of cholesteryl ester transfer protein (CETP) activity: review of in vitro and in vivo studies, Biochim Biophys Acta, 1994, 1215: 209-236.
69 Rubin, E. M. and Smith, D. J., Atherosclerosis in mice: getting to the heart of a polygenic disorder, Trends Genet, 1994, 10: 199-203.
70 Ferretti, G., Bacchetti, T., Negre-Salvayre, A., Salvayre, R., Dousset, N. and Curatola, G., Structural modifications of HDL and functional consequences, Atherosclerosis, 2006, 184: 1-7.
71 van der Steeg, W. A., Holme, I., Boekholdt, S. M., Larsen, M. L., Lindahl, C., Stroes, E. S., Tikkanen, M. J., Wareham, N. J., Faergeman, O., Olsson, A. G., Pedersen, T. R., Khaw, K. T. and Kastelein, J. J., High-density lipoprotein cholesterol, high-density lipoprotein particle size, and apolipoprotein A-I: significance for cardiovascular risk: the IDEAL and EPIC-Norfolk studies, J Am Coll Cardiol, 2008, 51: 634-642.
72 Sviridov, D. and Nestel, P., Dynamics of reverse cholesterol transport: protection against atherosclerosis, Atherosclerosis, 2002, 161: 245-254.
73 Stefan, N., Kantartzis, K. and Haring, H. U., Causes and metabolic consequences of Fatty liver, Endocr Rev, 2008, 29: 939-960.
74 Johnson, N. A. and George, J., Fitness versus fatness: moving beyond weight loss in nonalcoholic fatty liver disease, Hepatology, 52: 370-381.
75 Kantartzis, K., Thamer, C., Peter, A., Machann, J., Schick, F., Schraml, C., Konigsrainer, A., Konigsrainer, I., Krober, S., Niess, A., Fritsche, A., Haring, H. U. and Stefan, N., High cardiorespiratory fitness is an independent predictor of the reduction in liver fat during a lifestyle intervention in non-alcoholic fatty liver disease, Gut, 2009, 58: 1281-1288.
76 Vieira, V. J., Valentine, R. J., Wilund, K. R., Antao, N., Baynard, T. and Woods, J. A., Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice, Am J Physiol Endocrinol Metab, 2009, 296: E1164-1171.
77 Marques, C. M., Motta, V. F., Torres, T. S., Aguila, M. B. and Mandarim-de-Lacerda, C. A., Beneficial effects of exercise training (treadmill) on insulin resistance and nonalcoholic fatty liver disease in high-fat fed C57BL/6 mice, Braz J Med Biol Res, 43: 467-475.
78 Richard, D. and Trayhurn, P., Effect of exercise training on the rates of fatty acid synthesis in mice, Can J Physiol Pharmacol, 1984, 62: 695-699.
79 Johnson, N. A., Stannard, S. R. and Thompson, M. W., Muscle triglyceride and glycogen in endurance exercise: implications for performance, Sports Med, 2004, 34: 151-164.

APPENDICES

ENGLISH SUMMARY

The prevalence of the metabolic syndrome (MetS) has reached alarming dimensions in the Western World. MetS is comprised of a multitude of risk factors and can lead to serious complications like type 2 diabetes and atherosclerosis. Bile acid sequestrants (BAS), pharmacological agents that increase the fecal excretion of bile acids, and physical activity improve MetS and act beneficial in decreasing high blood glucose and high cholesterol levels, two components of MetS. However, underlying mechanisms are elusive. We evaluated whether the BAS and physical activity induced improvements in MetS are underlying specific changes in glucose-and cholesterol-handling in specific mouse models paralleling human disease.

We found that BAS decrease blood glucose levels by peripheral actions and not by modulations of liver glucose handling. Specifically, we found BAS to increase the clearance of blood glucose which coincided with a number of parameters indicating improved peripheral insulin sensitivity and glucose utilization. Additionally, we found BAS treatment to substantially reduce atherosclerosis development in atherosclerosis-prone mice which is most likely mediated by a switch from bodily cholesterol accumulation to cholesterol loss.

We further established that physical activity beneficially modulates cholesterol metabolism by enhancing its fecal excretion especially as bile acids but also as neutral sterols. This coincided with a reduced development of atherosclerosis and hepatic fat storage. Intriguingly, we found a negative correlation between the amount of physical activity and atherosclerosis development.

Altogether, the studies of this thesis provide novel insights on how BAS and physical activity beneficially affect MetS risk factors and its complications.

NEDERLANDSE SAMENVATTING

De toename in het vorkommen van het metabole syndroom (MetS) in de Westerse Wereld is alarmeerend. MetS omvat meerdere risicofactoren die kunnen leiden diabetes type 2 en atherosclerose. Galzout-bindende harsen (BAS), farmacologische stoffen, die de fecale uitscheiding van galzouten verhogen, en fysieke inspanning hebben een positieve invloed op hoge bloedsuiker spiegels en hoge cholesterol waarden in het bloed, twee belangrijke risico-factoren geassocieerd met MetS. Echter, de onderliggende mechanismen zijn onduidelijk. We hebben onderzocht hoe fysieke inspanning en BAS de genoemde verbeteringen in MetS veroorzaakten in specifieke muismodellen voor humane ziekten. BAS verlaagde de bloed glucose waarden door een reactie van de perifere weefsel, met name spier, en niet door veranderingen van het lever glucose metabolisme. We vonden dat BAS een aantal parameters van perifere insuline gevoeligheid en glucose verbruik verbetert. BAS behandeling leidt tot een substantiële verlaging van atherosclerose ontwikkeling in atherosclerose gevoelige muizen, waarschijnlijk veroorzaakt door een omschakeling van cholesterol ophoping naar cholesterol uitscheiding. We stelden vast dat lichamelijke activiteit het cholesterol metabolisme positief beïnvloedt door verhoogde fecale excretie van cholesterol, vooral in de vorm van galzouten maar ook als neutrale sterolen. Dit veroozaakte waarschijnlijk vermindering van atherosclerose en vet stapeling in de lever. Opvallend was de negatieve correlatie tussen mate van lichamelijke activiteit en ontwikkeling van atherosclerose. Samengevat geven de studies in dit proefschrift nieuwe inzichten in hoe BAS en lichamelijke activiteit de risico-factoren en complicaties van MetS positief kunnen beïnvloeden.

DEUTSCHE ZUSAMMENFASSUNG

Das metabolische Syndrom (MetS) hat Besorgnis-erregende Maße in der westlichen Welt erreicht. MetS wird durch eine Vielfalt von Risikofaktoren charakterisiert und kann zu ernsthaften Komplikationen wie Typ 2 Diabetes und Arteriosklerose führen. Gallensäurerhemmer (BAS), pharmakologische Mittel, die die fäkale Ausscheidung der Gallensäuren erhöhen und sportliche Aktivität verbessern MetS. Beide wirken vorteilhaft bei der Senkung hoher Blutglukose-und Cholesterin- Spiegel, zweier Merkmale des MetS. Doch die zugrunde liegenden Mechanismen sind nicht eindeutig. Wir untersuchten ob die durch BAS und durch körperliche Bewegung verursachten Verbesserungen in MetS durch spezifische Veränderungen im Glukose- und Cholesterin-Stoffwechsel hervorgerufen werden. Hierfür verwendeten wir spezifische Mäusemodelle, die menschliche Störungen des Mets wiederspiegeln. Wir fanden, dass die BAS-induzierte Senkung der Blutglukose durch Vorgänge im peripheren Gewebe, speziell in der Skelettmuskulatur, und nicht durch Modulation des Leber-Glukose-Stoffwechsels her*vor*gerufen wird. Im speziellen, BAS erzielt die Räumung von Glukose aus dem Blut, was mit Parametern zusammentraf, die auf eine verbesserte periphere Insulinempfindlichkeit und Glukoseverbrauch hinweisen. Zusätzlich führt BAS-Behandlung zu einer wesentlichen Verminderung in Arterioskleroseentwicklung in Arteriosklerose-empfindlichen Mäusen führte, höchstwahrscheinlich durch einen Wechsel von körperlicher Cholesterinansammlung zu Cholesterinverlust dur BAS. Der Cholesterinstoffwechsel wird weiterhin vorteilhaft von körperlicher Belastung beeinflusst denn körperliche Bewegung führt zu einer Erhöhung der fäkalen Cholesterinausscheidung insbesondere als Gallsäuren, aber auch als neutrale Sterolen,. Dieses traf zusammen mit einer verringerten Ausbildung von Arteriosklerose und Fettablagerung in der Leber. Außerdem fanden wir eine negative Korrelation zwischen Dauer der körperlicher Belastung und Arterioskleroseentwicklung. Insgesamt verleihen die Studien dieser Dissertation neue Einblicke darauf, wie BAS und körperliche Bewegung die Risikofaktoren und Komplikationen von MetS positiv beeinflussen.

*Herzlichen Dank an Angela und Wolfgang Meißner für kritisches Lesen der deutschen Zusammenfassung.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support of many people who contributed in different ways. To them I would like to convey my sincere appreciation. Contributions did not only come from scientific and academic sources but also from colleagues, family and friends who have shared experience with me during these past 4 years.

My promoters Folkert and Bert, I hope you enjoyed this experience as much as I did!
Folkert, you offered me a PhD position and welcomed me to the Laboratory of Pediatrics in 2006. Thank you for giving me the opportunity to learn and work in such a supportive environment; you sure know how to put a functional team together. I would like to thank you for your advice, critical comments, and for letting me "branch off" and experiment with the running wheels; it made an important difference in my work and I regained much needed enthusiasm.
I am also grateful for Bert's input to my supervision, joining with less than 2 years in my project left. Working with you was very stimulating and I appreciate your guidance, especially with the last two chapters of this thesis. Thank you for your valuable help during the last two years. I am also still wondering how you manage to bike on this very tall bike of yours that you provided for me when I was working at the AMC…

I would like to thank the members of my reading committee for revising my thesis: Prof. Peter Sauer, Prof. Gertjan van Dijk and Prof. Bart Staels.

My gratitude to my paranimfen Gemma Brufau and Milica Stankovic. Gemma, your mischievous behavior operating your bike up the steepest mountain of Groningen is forgiven. First because I busted your performance enhancing strategies and second because – other than that - you have been a pillar of support to me inside the lab and out of work, we shared great laughs, thoughts, wine* and I can always count on you! Milicita-ji, we first met dancing in the small $1m^2$ living room of Daniela. You have become an important friend to me, thank you for sharing your good energy and advice with me and being there in sun, rain and swimming pool. (Very curious about our djembe project!)

A special place in these acknowledgements to the people I had the pleasure to collaborate with: Hilde, I was fortunate to work together with you (Joint Task Force W!). Thanks for all the help you have given me in the first stages of my PhD, I have learned a lot from you. The nights at the Animal Facility and surviving the days with Ramon's Brazilian Corn chow and heavy metal tunes were bearable because we were hanging in there together. I would like to thank Elisa Lombardo

for taking time out in August 2009 to teach me valuable tools for my last two studies. Our working together also made a lasting impression at the AMC (…floor…). Good luck with finishing your PhD! Henk, thank you for your indispensable help and commitment to chapter 7 of this thesis, this chapter would not have been possible without your efforts. You are great to work with and so are your critical observations and sense of humor. We still have to present the NEW method to quantify feces production!! I would like to thank Rick for helping me with the difficult parts of the animal experiments and sharing his expert microsurgery fingers for the studies of this thesis. Rudolf (de Boer) aka: Sergeant by Night, although we could not find positive results echo-ing the "drosophila hearts" of my mice, I very much enjoyed working with you and appreciated the upbeat atmosphere and your prompt and critical comments! Uwe, thank you for your open ears, willingness to discuss, dry humor and championship ability to clear sweets from our office. Small Theo ☺, oooo what nightmares and joy we had with zillion of bloodspots and urine samples. I came to appreciate you as small Theo with special skills in difficult modeling and calculations, and you were forced to appreciate me, the "Meissner", with all my questions. It was fun working with you.
In memory I would like to express my gratitude to Henk Visser who helped me getting started with my initial project utilizing the doubly labeled water technique for measuring energy expenditure. You were a great and encouraging teacher and mentor to me in my first months as a PhD student, and I am grateful for having had the opportunity to work with you
This work would also not have been possible without the support of Hilde, El Senõr Renze, Albert (awesome feepje!), Vincent (thanks for all the checking!), (big) Theo, Ingrid, Annemieke (microsurgery team), Paul and Pim B (music man) for their support with my work.

Everyone at the CDP, thank you for your assistance. A special thanks for the daily enthusiam of Silvia and Hesther, nice to work with such upbeat people! Ramon, thanks for filling our bellies with Brazllian corn soup. Silvia and Yvonne, thanks for providing me with all the space for my last experiment. Wiebe, keep up the exercise!

A big thank you and smile to all the people from the Laboratory of Pediatrics, a BIG group, with whom I learned a lot. I would like to thank Esther (thanks for giving good advice and nice to explore life music with you), Anke (I am happy that boating remains fun!), Marije (special thanks to you, page 199 of this book would surely look very different without your input!), Niels, Marijke, Maaike (Let's practice some songs!), Aycha, Torsten, Leonie, Harmen, Frans S, Frans C, Willemien (thanks for checking up on me this summer), Dirk-Jan, Marjan, Jelske, Fjordor, Juul, Jelena, Jurre, Arne, Wytzke, Margot, Anniek, Gijs, Nicolette, Leonie, Henkjan and everyone else!! A big thank you to the people from my office who shared the

common frustration about our dysfunctional printer and kept in great physical condition by climbing all the way to the third floor: Jan-Freark (Many thanks for your input to our cookie jar. And…I am wondering when we can ride your car on the Herrestraat again?), Annelies (We still need to have THAT dinner ☺), Goli (coffee break?), Mariette, Yan, Thomas (I am still smelling that cheese…), Nynke, Marjolein, Sabina (Blitz I was happy to share the office such a positive person and your help when I first started working was indispensable.) and Krzysztof. Thanks to the colleagues that I may have forgotten to mention unintentionally.

Eryn (Northseajazz tradition!) and Carianne (how are the brothers?), I am glad our path crossed through Gecko and I am happy we are staying in touch…you are all pretty…gek, and that's great.

A special thanks to the Hunze Rowing Dollies whom I shared good adventures in and out of the boat: Elizabeth (Brinta Babe), Stijn, Nastasha, Anneke, Machteld, Klaartje, Willemien, Willemijn, Tsitske, Theo, Robert…racing with you was for sure a great way to improve my Dutch ☺.

To my fellow participants of the Intensive Ashtanga Teacher Training 2009/2010. Pfff…who knew what we had infront of us? It was great to make this journey with you all and it sure helped with my work. Special thanks to my buddy-ji Léonie, Nathalie, Babette and Rene.

Thanks to the individuals who contributed to my work because they inspired me in various ways: Anika Giehl, Elva Maxine Beach, Jason Winnick, Jill Davis, Yvonne Mielecke, Phil Schniter and Tim Kirby. And especially my mother.

To my friends in Groningen whom I could count on for their support: Daniela (I'm proud of you!), Fany (n'ice), Jelena & Dusko (thanks for your friendship, being the best neighbors, all the teas and stimulating conversations), Romy (Lampenfieber aus 1994 weg?), Goli, Sasja (cool to have an Ashtanga buddy in town) and the Stammtisch Pintelier: Ulli, Reinhard, Carlos, Arne: Prost!

I am grateful to all my friends who kept supporting me from far away, who never stopped inspiring me, who visited me and /or traveled with me during the last 4 years. I feel at home with y'all from distant places: Anne (Toelle): Woofs and Poodle-Power forever my dear friend whether in Texas, Sanibel, Mc-Gregor Blvd., Istanbul or G-city. Cheasty con mucho gusto no suggestion nicargyüse yoghurt, human pyramids and good wine. Judy and Ashmi, thanks for visiting numerous times, coming to Columbus for me, your creativeness and good vibes. I have good friends in you that I would not want to miss. Waqas ! Your good energy always carries over. Thanks for all the encouragement. You rock. Christine (for being a

goldmine of valuable advice), Kathrin (Wo ist Jutta?), all of Austin BT, Conny, Nicole and Julia.

Xavier, I am so happy that we met and to have the time with you is beautiful. A big warm thank you for your dear support in the last months.

Herzlichen Dank an meine Familie. Insbesondere:
Thomas, schön, daβ Du mein Bruder bist, auf Dich ist immer Verlaβ, und das hat mich oft beruhigt. Anja, mit Dir ist gut Kirschen essen! Danke für Eure Besuche in G-city und Euren Humor. Mutti & Vati, vielen Dank für Euer „Über-die-Schultern-spucken“, Eure Versuche zu verstehen was ich eigentlich mach und das Weitervermitteln der Meiβnerischen Zähheit. Ich bewundere wie Ihr mit den gegenwertigen Schwierigkeiten umgeht und wünsche mir noch viele schöne Zeiten mit Euch im schönen Schönow!

BIOGRAPHY

Maxi Meissner was born in the spring of 1980 in Berlin (GDR). A few years later, the family, comprised of Angela, a civil engineer, Wolfgang, a biology and chemistry teacher, and the two children, Thomas and Maxi, moved to Schwedt, a small town at the German-Polish border.

At the turn of the 19th century Schwedt was known for its tobacco production. In the 1960-ies when Schwedt was the end-point of a petrol pipeline coming from Russia, the town developed into a young industrial East-German model city. Quickly, whole new neighborhoods consisting of socialist apartment buildings emerged. Such apartments had good sanitary conditions and were highly desirable, also to the Meißners.

Maxi's affinity for physical activity was sparked early. As part of East-German ideology, children were screened for their potential at different sports. Like her brother Thomas, Maxi was invited to try the sport of rowing at the age of 8. To Maxi, staying afloat in a rowing shell seemed more fun than spending time with her violin. As the years passed, Maxi got to know every cubic m spanning the picturesque shoreline of the Friedrichsthaler Wasserstrasse. At the age of 15 she took the opportunity to explore new waters and moved to Potsdam where she attended the Eliteschule des Sports and moved herself in a boat up and down the river Havel. Then and there Maxi's interest for exercise physiology manifested. She wondered why the sports scientists pricked her ear for blood so frequently that it often turned purple. Fascinated by their work, Maxi also realized that their training advice was worthwhile to follow; she won gold at the Junior-World Championships twice.

Luckily, for Maxi, who wanted to travel, the walls around East Germany had long disappeared when in 1998 she was recruited for rowing by American universities. At the age of 19, she started studies in exercise physiology at The Ohio State University in Columbus, Ohio. During the next four years, Maxi experienced the "American way of life". The days were very long with the first rowing practice starting at 5:30 and so much to do. Maxi realized that 5 hours of sleep is not good and was happy when her contract as a rower, her time as a

student neared its end and the blisters on her hand developed into smooth skin. After gaining experience working in cardiac and pulmonary rehabilitation at the Ohio State Hospitals Center for Wellness and Prevention, Maxi's interests switched from exercise performance towards physical activity in disease prevention and therapy.

To pursue a masters degree in exercise physiology, focusing on cardiac metabolism, she moved to Austin, Texas at the age of 24. She was fascinated with her studies and her curiosity about the interactions of lifestyle and disease grew deeper. Austin with its creative inhabitants, abundant natural beauties, its independent arts and music scene, Austin BT and its good weather year around was a love affair at first sight.

However, wondering whether she liked living in Europe, close to her family, Maxi decided to search for a PhD-position in Europe. In the fall of 2006, she took on a PhD-position ("The role of Fxr in energy expenditure") at the Laboratory of Pediatrics at the University of Groningen where the working environment appeared perfect for learning and growing while tasting European life and being closer to family.

During her stay in Groningen Maxi not only enjoyed the ups and downs of being a PhD student, but learned that balance is important for well-being. In her work she missed the direct application of her work to people and decided to fill that desire by transferring her enthusiasm for ashtanga yoga onto others in her free time. In May 2010, Maxi became a certified ashtanga yoga teacher and now enjoys teaching ashtanga to anyone who wants to learn (www.moxieyoga.com).

After putting down the pipettes, Maxi enjoys swimming in open water, being in nature, checking out life music, reading, playing the djembe (badly), traveling, meeting people, the brain science podcast and avocados. In the future, Maxi's work will continue to contribute to society's well-being at large; in a basic way by investigating effects of lifestyle on aging and in an applied way by teaching yoga.

LIST OF PUBLICATIONS

Bile salt sequestration induces hepatic de novo lipogenesis through farnesoid X receptor- and liver X receptor alpha-controlled metabolic pathways in mice. Herrema H, Meissner M, van Dijk TH, Brufau G, Boverhof R, Oosterveer MH, Reijngoud DJ, Müller M, Stellaard F, Groen AK, Kuipers F.
Hepatology. 2010 Mar;51(3):806-16.

Exercise enhances whole-body cholesterol turnover in mice. Meissner M, Havinga R, Boverhof R, Kema I, Groen AK, Kuipers F.
Med Sci Sports Exerc. 2010 Aug;42(8):1460-8.

Voluntary exercise increases cholesterol efflux but not macrophage reverse cholesterol transport *in vivo* in mice. Meissner M, Nijstad N, Kuipers F, Tietge UJF.
Nutr Metab (Lond). 2010 Jul 1;7:54.

Bile acid sequestration reduces plasma glucose levels in *db/db* mice by increasing
the metabolic clearance of glucose. Meissner M, Herrema H, Dijk v.TH, Gerding A, Havinga R, Boer T, Müller M, Reijngoud D-J, Groen, AK, Kuipers F.
Submitted

Voluntary wheel running decreases atherosclerosis development and increases sterol excretion in hypercholesterolemic mice. Meissner M, Lombardo E, Havinga R, Tietge UJF, Kuipers F, Groen AK.
Submitted

Beneficial effects of bile acid sequestration and voluntary wheel running on cholesterol turnover and atherosclerosis in hyperscholesterolemic mice. Meissner M, Wolters H, Boer d RA, Havinga R, Boverhof R, Bloks VW, Kuipers F, Groen AK.
Submitted